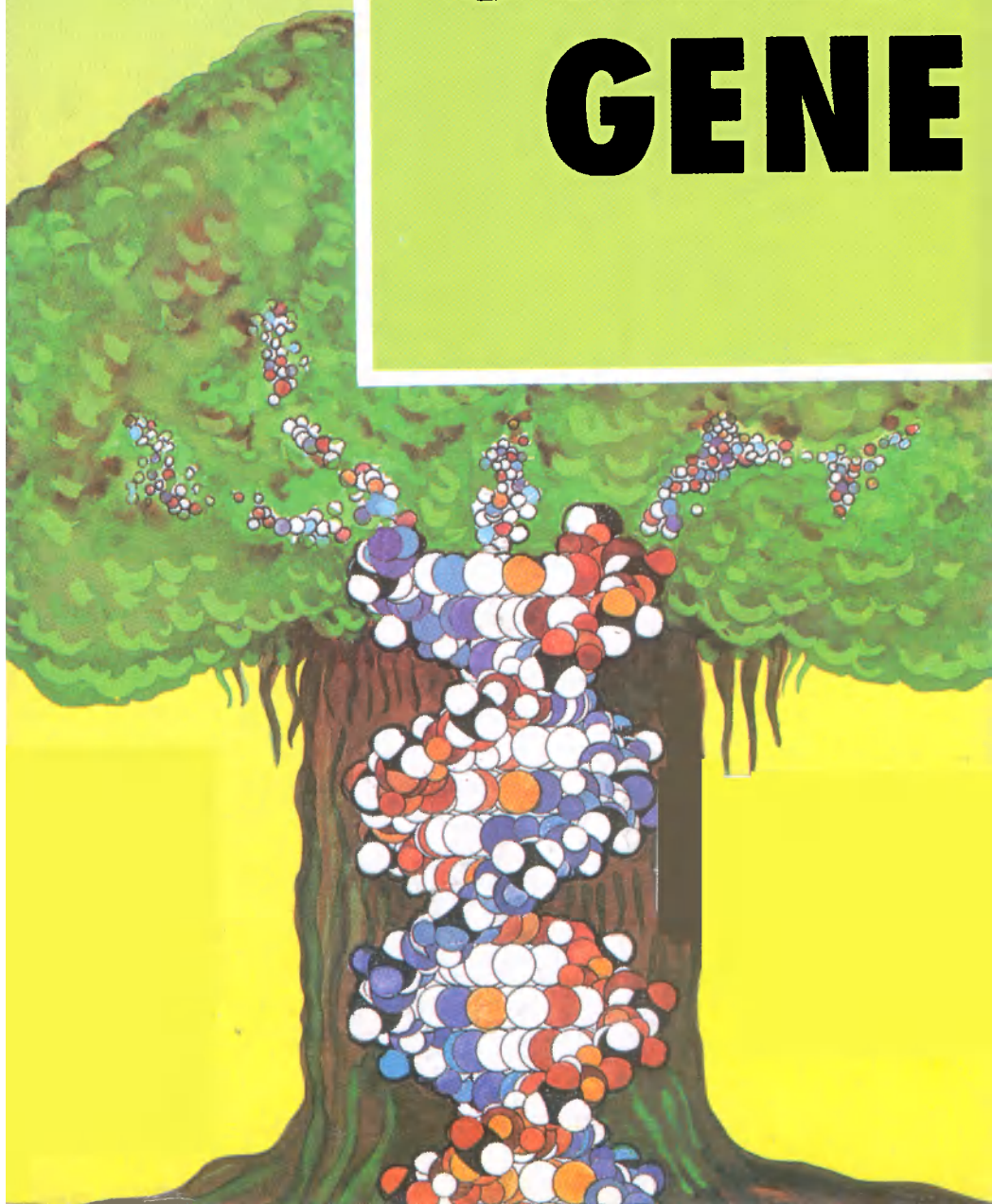


SHAKUNTALA
BHATTACHARYA

GREEN GENE



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GREEN GENE

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India

Green Gene

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FOREWORD

Evidence from the Middle and Far East shows that humans started growing crops some ten thousand years back. This was followed by domestication of animals for food, transport and tillage. Man also exploited microorganisms for producing bread, fermented foods and drinks, without understanding the processes involved. Initially, in the absence of the understanding of the basis of heredity and variation, the improvements in crops and animals following their domestication were slow.

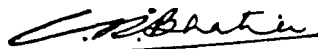
The principles of genetics were formulated by an Augustinian monk, Gregor Mendel, who carried out experiments on the garden pea plant. The original paper published in 1865 remained in obscurity till it was rediscovered at the turn of the century. The structure of DNA, — the genetic material determined in the mid- fifties, is regarded as the most significant development in life sciences. This was followed by the deciphering of the genetic code. These discoveries laid the foundations of molecular biology. The applications of the knowledge and techniques of molecular biology for the improvement of microbes, plants and animals is now widely recognised as biotechnology. Recombinant DNA techniques have made it possible to introduce and express the genes of choice from any living organism in the desired microbe, plant or animal.

Classical genetics and manipulation of genes at the cellular level have played a major role in enhancing the productivity of plants and animals. The molecular manipulations of the genetic material are likely to play an even greater role towards increasing the efficiency of microbial, plant and animal production systems. Genes for fixation of atmospheric nitrogen have been transferred to microbes other than those naturally endowed with them. Environmentally friendly microbes, blue green algae, mycorrhizae and earth worms are being used to increase soil fertility. Microbial and natural plant products are

being used for insect pest control in integrated pest management. Genes for toxic proteins from microbes and even of scorpion venom have been introduced into crop plants for protecting them from insect pests.

This has greatly enhanced the value of rapidly dwindling biodiversity which represents the unique combination of genes in each of the species as a result of natural evolution. Seed and gene banks, and wild life reserves are being set up all over the world to conserve the available biodiversity. *In vitro* propagation of the endangered plant species are being used to bring them back from the brink of extinction.

Making use of the expertise available in the country, the Publications and Information Directorate (PID) is bringing out a series of popular monographs in biotechnology as a part of the Project on "Dissemination of Biotechnology Information" sponsored by the Department of Biotechnology, Government of India. These monographs would benefit school, college and university students and teachers as well as members of the public and create an awareness among them towards the newer developments in this field taking place globally and in the country. They would also create awareness of the enormous potential of biotechnology in improving our socio-economic conditions. Both PID, which is one of the premier institutions of its type in the country engaged in dissemination of scientific information and science popularization for more than 25 years and the authors of the various monographs, who are all highly acclaimed for their scientific contributions have joined hands in this very important venture. I am confident that the readers would find the monographs informative and enlightening and this would contribute to the development of the multi-disciplinary area of biotechnology in the country .



(C.R.Bhatia)

Secretary to the Govt. of India
Department of Biotechnology

PREFACE

In the last few years, Biotechnology has become a household word. Yet, very few understand how wide a range of technologies this covers. Whenever I have talked of Biotechnology in agriculture to my post-graduate students, they have always said that it means the genetic engineering of crops. They were surprised to learn about the large vista that agricultural biotechnology encompasses. From such low-tech as vermi-culture and biofertilizers to the high-tech genetically engineered plant that produce animal proteins. It is this wide range of biotechnology in agriculture that I have tried to present for the layman and the budding scientists. If they can imbibe some of the excitement of this fast-developing field my purpose would be fulfilled.

ACKNOWLEDGEMENTS

Sitting down to put one's disorganized knowledge and thought is a hard enough task, but doing it for the layman is daunting. Dr. Bal Phondke with his usual charming persistence finally broke down my resistance to putting pen to paper, and now that the book is ready, he is the one who deserves my heartfelt thanks. Besides the above, he along with Dr Sukanya Datta did a wonderful job of editing my manuscript.

Others at PID have contributed much to the book. To all of them I am grateful. Lastly, I would like to thank my better half for sharing his computer with me, and putting up with my idiosyncrasies during the period of writing, and lastly for converting my computer "mess" into a presentable manuscript.

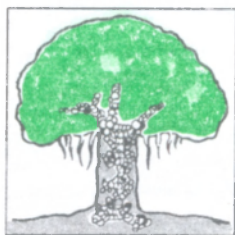
Dedicated
to my father
who encouraged all my creative activities

Contents

Changing face	...	1
Better and faster	...	10
Back to the future	...	24
Engineering better plants	...	29
Good riddance	...	49
How safe is safe?	...	63
The natural way	...	74
Future perfect	...	92
Glossary	...	103

Agriculture which formed the base of human civilization, has always awakened the creative genius in human beings. This is because it provides for the basic utilities of life — food, fuel and clothing. As human population goes on increasing, ways to get the maximum out of the limited land area available for cultivation pose the greatest challenge to humankind.

Starting with the expertise of prehistoric women who foraged for food and grains and ultimately started what we now call the science of agriculture, the effort has always been



Changing face

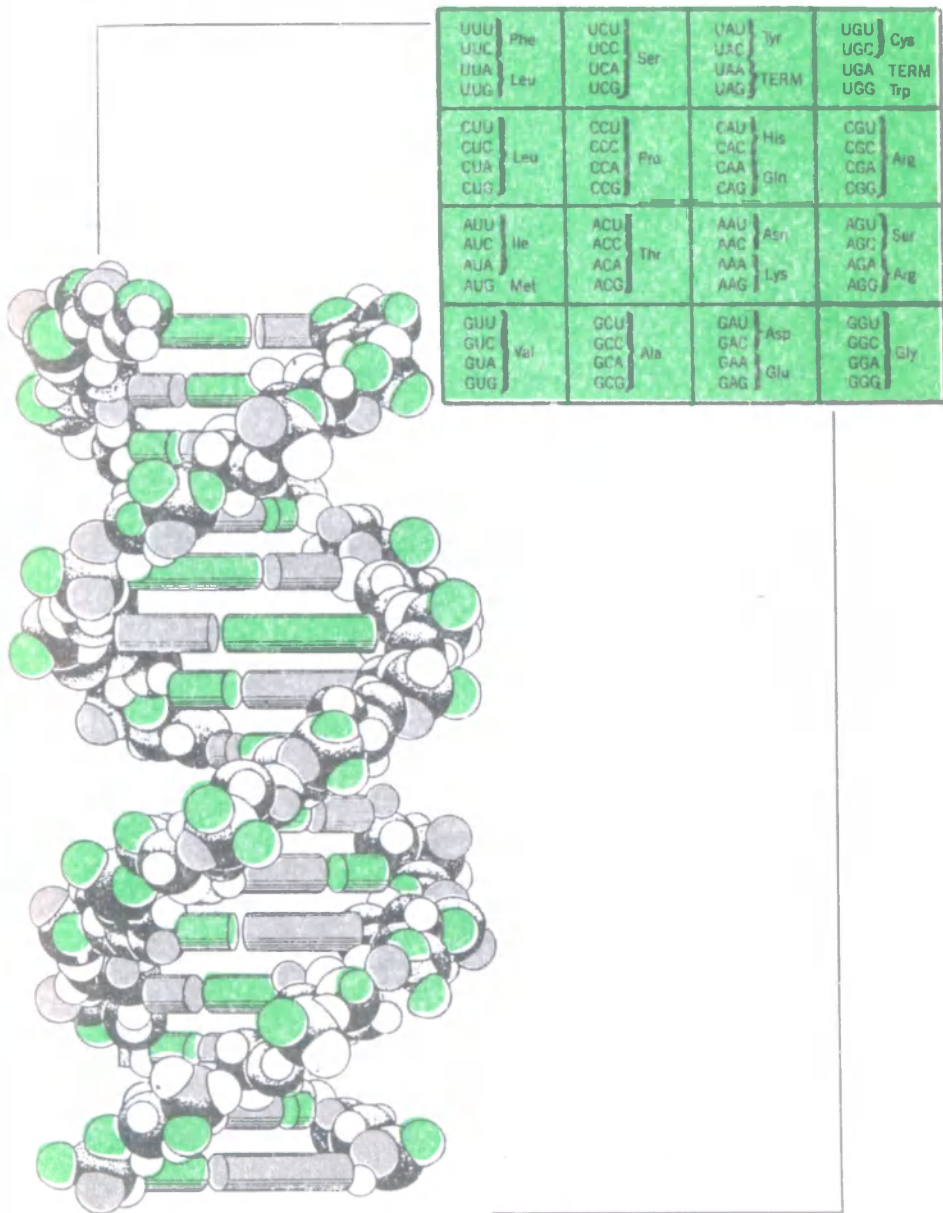
geared towards increasing yield and improving quality. Progress, however, was very slow as the science behind agriculture was not understood till the beginning of this century. In the late nineteenth century came the discovery that crops required mineral nutrition and this laid the foundation for improving yield with the addition of fertilizers. The rediscovery of Gregor Mendel's work on the crossbreeding of peas led to the widespread use of hybridization to produce improved varieties. As the understanding of the hereditary mechanism began to grow, many new methods such as inducing mutations to get a wide range of variations from which to select improved varieties, were exploited. The selection process, however, continued to be extremely long drawn and unpredictable. It took over fifteen years before a suitable variety could be thus selected for release to the farmer.

During the period 1930-1970 exciting discoveries were being made by the scientists, and in the years to follow these



changed the face of agriculture. The first of these was the discovery that deoxyribonucleic acid (DNA) is the hereditary material in living organisms. This was followed by the establishment of the structure of DNA by James Watson and Francis Crick in 1953 and the understanding of the genetic code. The genetic code is the way that the hereditary information stored in DNA is translated into proteins, whose basic structure consists of a chain of amino acids. Proteins in their turn perform as enzymes to catalyse the reactions which lead to the complex organisation, metabolism and reproduction of a living cell.

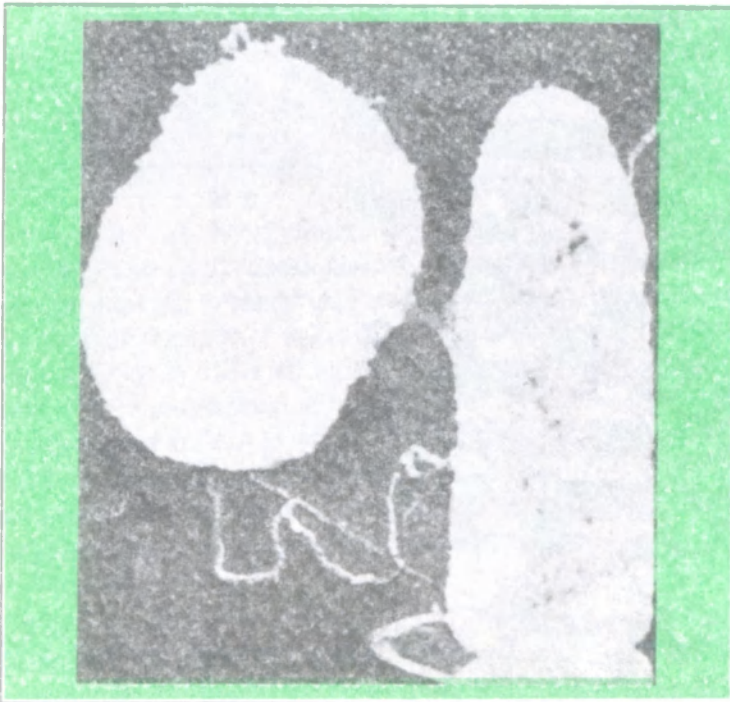
DNA consists of a backbone of deoxyribose sugar and phosphate on which are arranged four bases or nucleotides — adenine (A), thymine (T), cytosine (C) and guanine (G). The DNA molecule is a double helix consisting of two polynucleotide chains joined by hydrogen bonds between the base



The genetic code deciphered

pairs A-T and G-C. During replication of DNA each chain acts as a template for a new chain. When the DNA is translated into proteins, a set of three bases (triplet) acts as a code for a single amino acid. There are in most cases more than one code for a single amino acid. The mediation between DNA which is located in the nucleus and the proteins which are synthesised in the cytoplasm is through the ribonucleic acids (RNA). RNA differs from DNA in being single stranded and in having ribose instead of deoxyribose as the sugar and uracil (U) instead of thymine as a base.

Genes govern the hereditary characters. A gene is a stretch of DNA which codes for a particular protein. It also includes certain portions of DNA which are responsible for



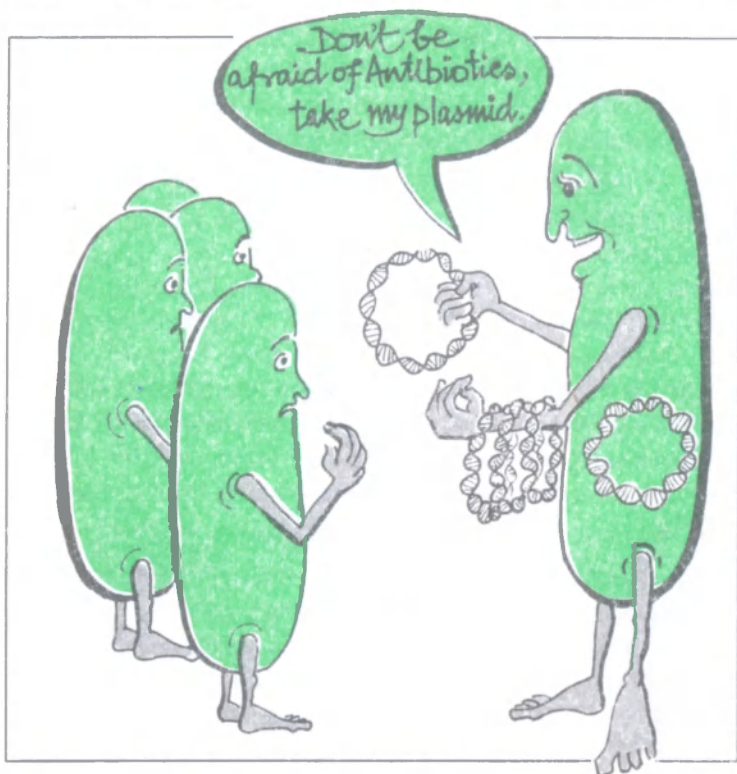
Conjugation in bacteria

turning the genes on and off. The switch is required as all genes do not function at all times during the life of a cell. Modern technology has helped in the isolation and identification of genes for a large number of proteins.

One of the major discoveries which paved the way for **genetic engineering** or **recombinant DNA** technology was the discovery in 1952 by Joshua Lederburg of U.S.A. that bacteria conjugate and exchange genetic material. He observed that there were two populations of bacteria namely, M and F. The F contain a body which he called 'plasmid'. During conjugation F-bacteria pass the plasmid on to M-bacteria. In the following year, William Hayes established that the plasmid was made up of DNA. Plasmids thus were rings of DNA in the cytoplasm of bacteria and were distinct from chromosomal DNA.

Throughout the 1940's and 50's it was becoming apparent that pathogenic bacteria were increasingly gaining resistance to sulpha drugs and **antibiotics**. In 1959 Japanese scientists reported that genes for drug resistance were carried on plasmids, and these could be passed on from bacterium to bacterium. Within the bacteria, plasmids multiplied, so that each bacterium had multiple copies, enough to pass on some to other bacteria while still retaining their capacity for resistance. This capacity of plasmids to move from one bacterium to another would, in the years to come, be exploited for transferring genes from one organism to another.

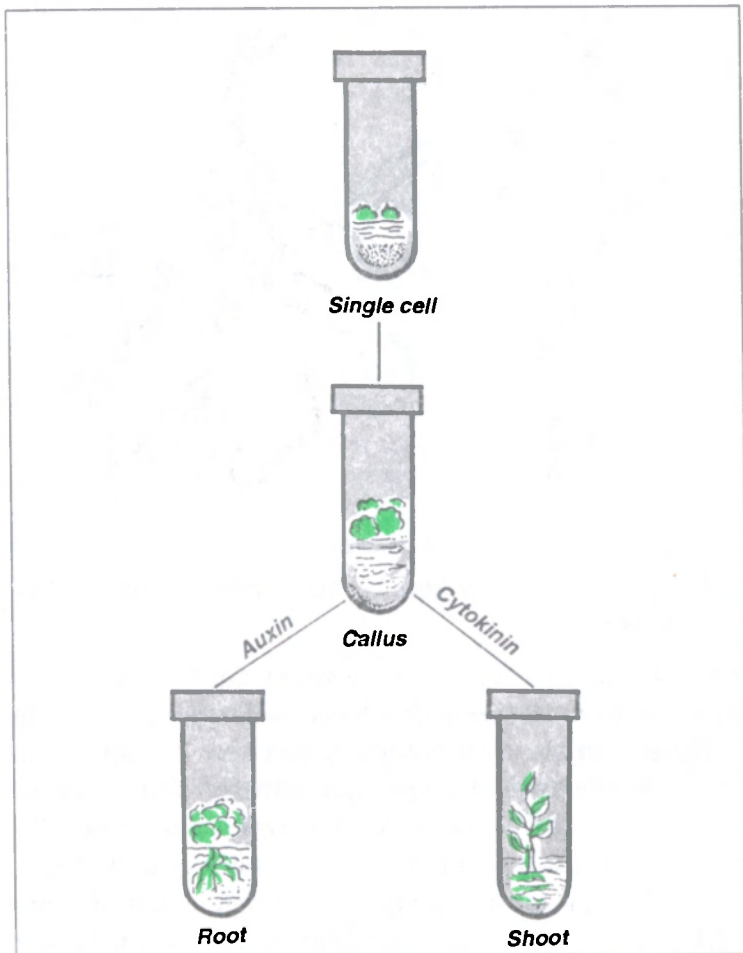
Another development taking place during this same period was the culture of plant cells. The first such culture having unlimited growth under aseptic conditions in a test tube was of phloem cells from carrot roots. These tissue explants when placed in nutrient medium containing essential salts, sugars and vitamins and proper stimuli could grow and divide. The effective stimuli that released this fast though unorganised, growth were those contained in natural liquid endosperm of coconut (what we drink as green coconut water), or certain other similar sources. The next attempts were to identify the



stimulating substances which turned out to be the plant growth regulators known as auxins and cytokinins.

In a series of classic experiments, F. Skoog and C.O. Miller, demonstrated that by change in concentration of auxins and cytokinins it is possible to dedifferentiate the **callus** tissue into root and shoot. It was observed that if cytokinin concentration was high then shoots would develop from the callus tissue, if the auxin concentration was higher then roots would develop.

The third landmark in the culture of plant cells came when it was realized that rapidly proliferating small carrot explants, growing at their optimal rates in slowly rotating flasks, release free and viable cells to the ambient medium. These cells did not look like the original cells from which they grew but



Cell culture techniques

survived, divided and grew to produce unorganised cultures of cells or organised cultures of cells which resembled an embryo. These were called somatic embryos and could give rise to normal carrot plants. This path-breaking experiment of F.C. Steward and his collaborators in establishing **totipotency** of plant cells laid the foundation for the micropropagation of plants from tissue cultures, and provided a means to



Galls on plants

obtain source material for genetic manipulation under controlled conditions.

Simultaneously with the work being done on plant cell cultures, another dramatic discovery was being unfolded by A.C. Braun and his collaborators. Braun from the start of his career was interested in crown **gall** tumours and its causal agent, the bacteria *Agrobacterium tumefaciens*. During the early period of his work he found the sunflower to be highly suitable material for the study. Surprisingly some of these tumours did not contain the bacterium and these gall tissues could be grown and multiplied in culture. What was, however, most interesting was the fact that a year later, pieces of this culture when implanted into normal sunflower yielded typical crown gall tumours, although once again no *Agrobacterium* was found in these tumours. It was therefore presumed that the bacteria had caused heritable genetic changes in the tumour tissue. A quarter of a century was to elapse before the cause of this genetic transformation could be identified.

In addition to the normal chromosomal DNA, *Agrobacterium tumefaciens* contains small circular double-stranded DNA called plasmids. The tumour causing ability resides in a tumour inducing plasmid called Ti.

When a plant is injured it produces compounds which attract the *Agrobacterium* from the soil. During infection, the bacteria invade the cells and grow in it. Some of the bacteria lyse thus releasing their DNA in the cell. From this point, the presence of bacteria is no longer necessary for tumour formation. By an unknown mechanism, a small fragment of the Ti plasmid, containing the genes for replication becomes integrated into the chromosomes of the host. The integrated fragment breaks down the hormonally regulated system that controls cell division and the cell is converted into a tumour cell. The auxin metabolism is the main target. In 1986, Braun observed, "the Ti plasmid DNAs carried by the inciting bacteria of the crown gall disease have served in the recent past and will doubtless continue to serve, as a vector for the introduction of new and desirable genetic information for genetic engineering studies designed for crop improvement." This applied aspect of the problem has led to the development of new industries. Recent estimates suggest that the agricultural potential of this new technology is so great that it will lead to the development of a multi-billion dollar industry world-wide by the end of the present century.



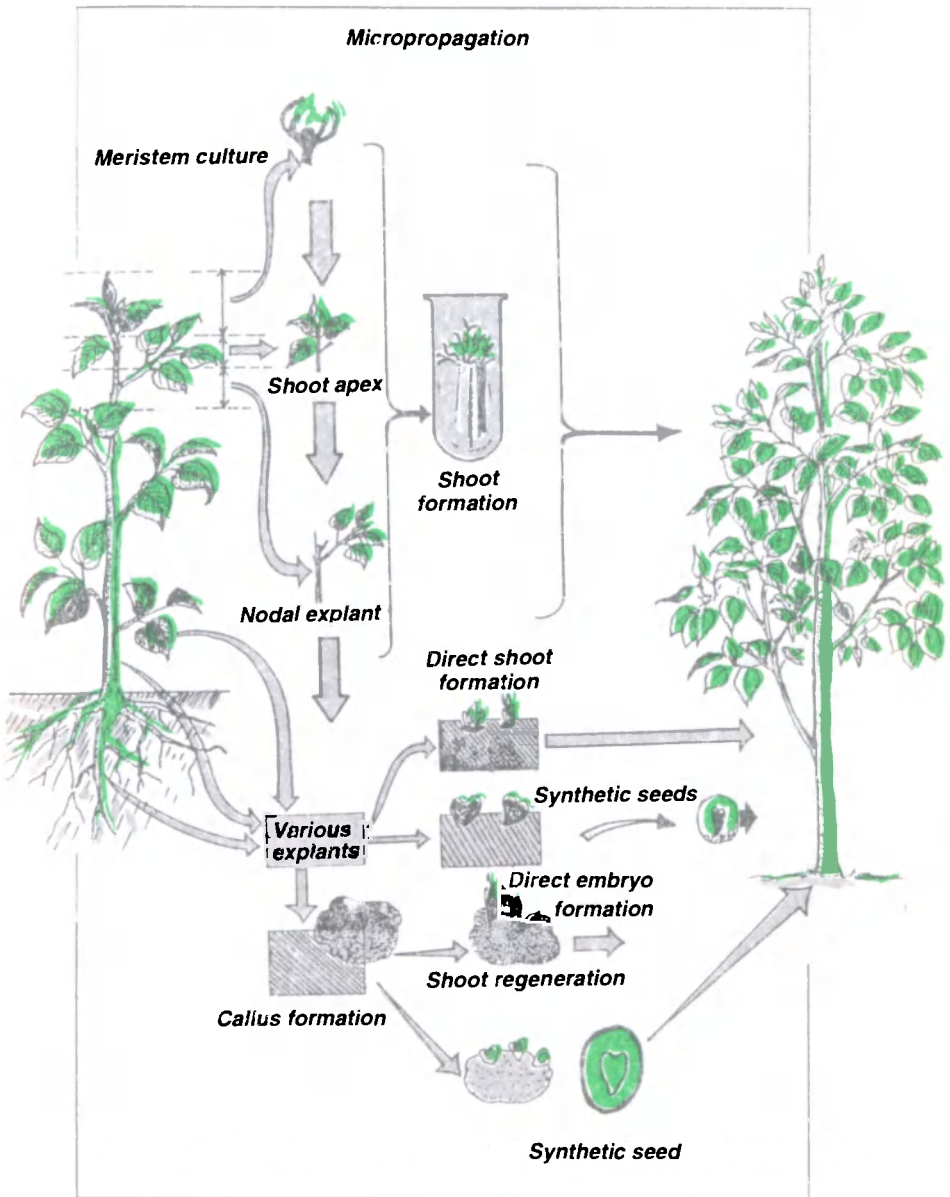
In order to produce higher agricultural yields not only do we have to produce better quality of plants, but also produce them in large enough numbers. The common banana, which if multiplied from a single plant would need years to produce a million offshoots, given that a plant produces 2-3 offshoots per year. But if one uses a rhizome and advanced tissue culture techniques, one can get 40 seedlings per explant in three weeks. Each of these seedlings in their turn can multiply at the same rate to produce 1600 plants in another three weeks. Two more rounds and within a period



**Better and
faster**

of three months one is able to produce approximately 2.5 million seedlings from a single parent. These techniques have made the micropropagation of plants a lucrative venture and many companies are cashing in on it.

Large scale plant regeneration using tissue culture method is quite widespread. The three basic techniques in use are shoot apex propagation, organogenesis and embryogenesis. The choice of technique depends on the end use and the species of plant. Certain procedures are, however, common to the three techniques. The culture medium commonly used consists of nutrients along with growth regulators such as auxins and cytokinins. Other additives used include coconut water. The medium is solidified using agar. The composition of the culture medium may be varied to suit the individual requirements of the species. The bits of tissue to be cultured are made germ-free by dipping into alcohol and then transferred aseptically into test tubes or flasks containing sterile



medium. They are then kept under adequate light in a temperature controlled room for growth and subsequent reculture.

Shoot apex propagation is used for ornamental plants and for parental breeding lines. Maintenance of the latter is usually time consuming and difficult by standard methods. There are three broad stages for production of plantlets from the shoot apex. These stages require alteration of culture medium and growth conditions. The first is when the tissue is established in culture. The second and the most important stage is the production of multiple shoots. Stage three is root formation and conditioning of the plantlets prior to transfer in the field.

The transition stage from the laboratory to the field is a critical period. Mass application of micropropagation rests on the ease with which the plantlets can be induced to grow in soil in a normal environment and also on their ultimate survival rate.

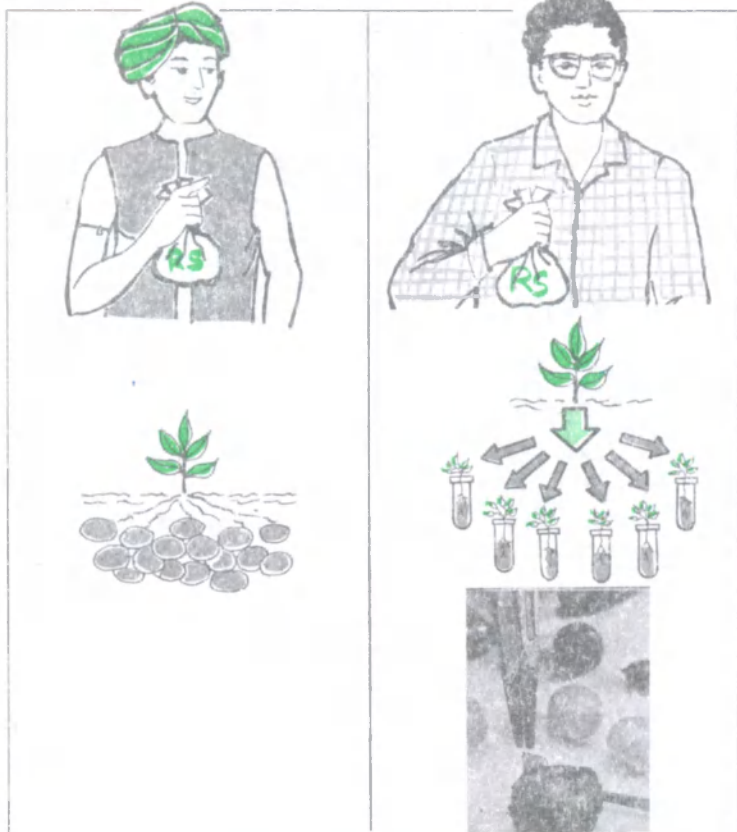
Three shocks await the plantlets. They must now make their own food, and fight infections and desiccation. However, infections can easily be avoided by sterilizing the soil and the pots and most farmers willingly lend a helping hand. Desiccation, however, is a major block. Excessively high water loss is reported for most species immediately after transplantation. This is because the plantlets have been raised in high humidity condition and have reduced cuticular wax covering, as well as a cellular architecture containing much intercellular spaces. These lead to a high rate of evaporation. In normal plants there are small openings called stomata on the leaves. The opening and closing of the stomata controls the rate of water loss from plants. In tissue cultured plants, however, the stomata are slow to respond. To add to these problems is the fact that in regenerated plants the connection between xylem, the water conducting tissues of the roots and those of the shoot are inadequate. The transplanted plantlets are therefore kept under a condition of high humidity by covering these



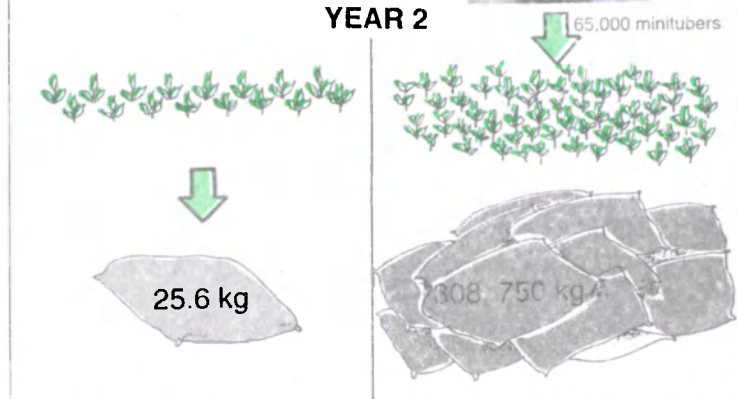
with plastic. The humidity is gradually adjusted to the normal level, and the 'hardened' plantlets become the equivalent of normal seedlings, ready for transplantation in the field.

Plants grown from shoot apex cultures are genetically stable. **Clonal** propagation is routinely used to multiply existing ornamentals cheaply. It is also used to maintain unique parents such as male sterile lines used for the production of hybrids, and also to multiply hybrid plants which are normally sterile as for example petunia. Normally the shoot tip and axillary buds are used. A slightly different approach to shoot

YEAR 1

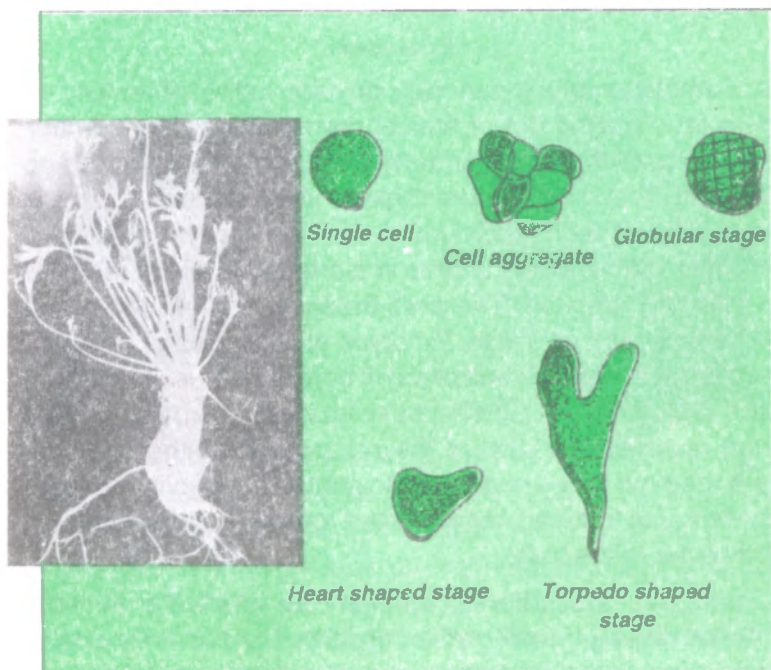


YEAR 2

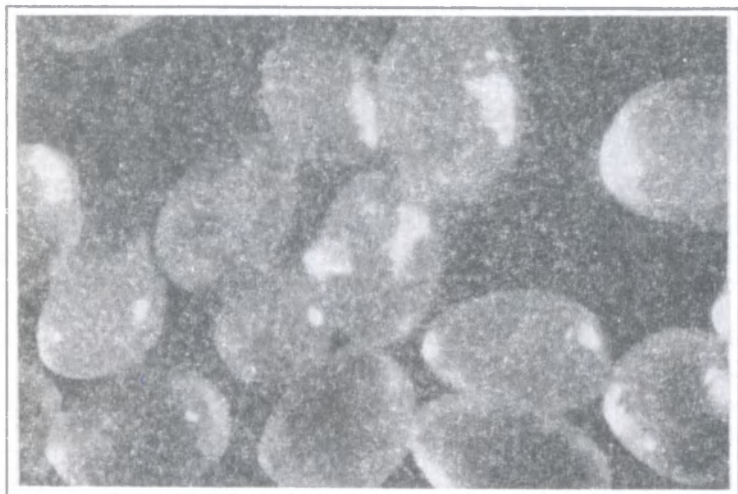


apex culture has been successful enough with potatoes to be commercialised. This is the production of minitubers. When axillary shoot cultures of potato are raised in a medium containing appropriate levels of the plant growth regulators, gibberellic acid and cytokinin, they form a large number of tiny tubers. These minitubers, when planted in the field generate normal plants. The cost effectiveness of this method has deservedly made it a hot favourite of potato farmers.

Plantlets can also be raised by culturing tissues such as leaf explants and callus tissue. In organogenesis shoot formation is followed by root formation. When the roots and shoots develop simultaneously the process is known as embryogenesis. The commonly used culture medium has to be fortified with iron as this is very necessary for embryogene-



Somatic embryogenesis. Carrot seedling germinating from an artificial seed (Inset)



Artificial seeds

sis. Somatic embryos generally develop in the same way as an embryo arising out of sexual reproduction. The somatic embryos pass through a globular stage, a heart-shaped stage and finally reach the mature torpedo shaped stage. Cultures are usually started on solid medium, and the callus so formed is transferred to liquid cultures in bioreactors where they are aerated and agitated. The cells breaking off from the callus differentiate into embryos. Embryos at all stages of development as also single cells broken off from the callus are present in the liquid medium. Sorting these is one of the major hurdles in the commercialization of the process. Once the embryos are sorted out they are allowed to mature on a solid medium. Dormancy is induced before these embryos are processed for transfer to the field.

There are four different methods by which the somatic embryos can be transferred to the field. The first is by germinating them in the laboratory and then transplanting them in pots in greenhouses before transferring them to the field. A second method involves encapsulation of individual embryos



in a gel containing adequate nutrition for the embryo. Such 'artificial seeds' can then be planted directly in the field. The third method which is being explored on a large scale is fluid drilling. The method involves producing germinated embryos under controlled conditions, mixing the selected seedlings in a gel-like medium and finally sowing using a fluid drill. The gel used is somewhat like agar and can be fortified with nutrients, pesticides, growth regulators and any other necessary chemicals. Fluid drilling of pre-germinated embryos have

already been developed for a number of crops including tomato, carrot, cabbage, cucumber and lettuce.

Embryo-culture has had a long history and even a hundred years back scientists had attempted to culture embryos in well-defined sterile media. The main objective was to explore nutritional requirements of plant development. The basic process remains unchanged to this day. Mature embryos require only inorganic salts and sugar, but the more immature the embryo, the more complex the medium required for its growth. The major uses of this technique in agriculture are rescuing incompatible hybrid crosses, clonal micropropagation and overcoming seed dormancy and seed sterility.

In crop plants interspecific and intergeneric crosses are frequently attempted to introduce desirable characteristics such as disease resistance. Incompatibility is frequent in such crosses and results in shrivelled seeds with aborted embryos. Non-viable seeds may result if there is low endosperm (food storage tissue) to nucleus ratio. It may also result if the endosperm fails to develop as this leads to starvation and abortion of the embryo. A third reason is embryo-endosperm incompatibility where the well-developed endosperm produces toxins that kill the embryo.

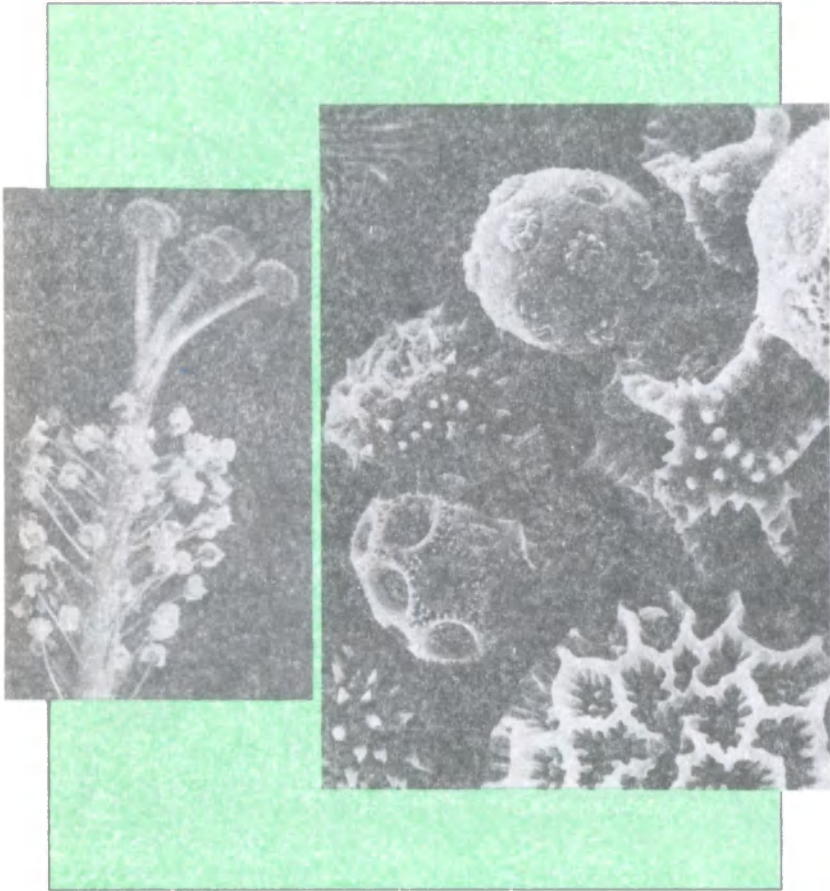
Culture of hybrid embryos prior to abortion is a way to overcome the barriers which arise after the ovule has been fertilized by the pollen. The stage at which the embryo is excised is critical. Waiting for the embryo to mature may result in abortion. At the same time, the more immature an embryo, the more difficult it is to culture because of its complex nutritional requirements. However, hundreds of incompatible crosses have been rescued by this method. For instance, in India seed-shatter resistance was transferred from the Indian mustard to rape seed by this technique.

The second use of embryo rescue is for clonal micropropagation. Embryos have a high regenerative potential because of their juvenile nature. The method is particularly suitable for



conifers and cereals. Embryo rescue technique is also used to overcome seed dormancy and seed sterility. Dormant seeds are viable but do not germinate because of the presence of chemical inhibitors or mechanical resistance of the structures covering the embryo. Culturing excised embryos may bypass dormancy of these species.

In the Philippines and Thailand a '*Macapuno*' or '*Kati*' coconut fetches twenty times the price of an ordinary green coconut. *Macapuno* coconuts are characterised by a soft endosperm which fills the whole nut resulting in a creamy



Pollen grains of different species through the electron microscope as compared to pollen grains as the eye sees it coconut water. These nuts fail to germinate because the endosperm rots before germinating embryo can grow out of the shell. The plants are heterozygous for this recessive trait or in other words only 25 per cent of the fruits will be '*macapuno*'. If seedlings can be produced from the '*macapuno*' nuts by an embryo rescue technique, then all the plants so produced will be homozygous for the recessive trait and a

hundred per cent '*macapuno*' yield will be assured by self pollination.

The plant's equivalent of the sperm is the pollen. Pollen biotechnology is the manipulation of the different aspects of the pollen biology for production of better crops. These include the screening of pollen for desirable genes, overcoming barriers to cross fertilization and production of haploids.

Screening pollen for desirable genes is dependent on whether these genes produce identifiable proteins in the pollen. Research has shown that traits such as resistance to diseases and tolerance to salinity, temperature, herbicide and water stress are expressed in pollen as well as parent plants. Thus plants resistant to a particular stress may be identified by studying the germination of the pollen from the plant under stress conditions. For instance pollen grains which germinate in the presence of a toxin produced by a pathogen identifies a disease resistant plant. Pollen from a susceptible plant would fail to germinate under these conditions. Use of pollen for screening has many advantages. The technique is fast and economical too. Since a single plant produces millions of pollen grains, a range of factors can be tested on a single plant.

Wild species often have traits which a farmer would like in his cultivars, but cross pollination is often difficult. The barrier is mainly due to the flowering of the two species at two different times, or other built-in fertilization barriers. Techniques for storing pollen under low temperatures or in solvents in which they remain viable over long periods have been developed. This pollen can be used to pollinate the female plants when they bloom.

Prefertilization barrier is mainly the inability of the pollen tubes to reach the ovule. This can happen due to inhibition of pollen germination by substances produced by the stigma or the inability of the pollen tube to traverse the style and reach the ovules. A number of techniques including *in vitro*



October 31, 1964

NATURE

In vitro Production of Embryos from Anthers of *Datura*

OBSERVATIONS of natural phenomena such as adventive embryony and production of plantlets from the leaf margins of *Bryophyllum* support the view that although cells may differentiate and appear to behave differently, their genetic potentialities remain the same. As a corollary to this view, differentiation operates not through a segregation mechanism but due to selective and programmed suppression or stimulation of genic activity.

In recent years this view has been greatly strengthened by the use of *in vitro* culture technique, and the regenera-

SIPRA GUHA
S. C. MAHESHWARI

Department of Botany,
University of Delhi,
India.

Sipra Guha and Satish Maheswari produced
the first haploid plants

fertilization have been developed to combat the problem. *In vitro* fertilization is effected through culturing of ovules and pollen grains together on a nutrient medium. The pollen germinates and enters the ovules. The fertilized ovules develop into seeds. Handling of individual ovules is tricky and time consuming. The technique has been modified so that instead of single ovules, bunches of ovules attached to the placenta are placed on the nutrient medium and dusted with pollen. The technique is called 'placental pollination' and is more effective since damage to ovules is minimised.

The first successful anther culture technique to produce haploid plants was reported by Indian scientists, Sipra Guha and Satish Maheswari in 1964. Since then, anther cultures have been used to produce haploid plants of many crops. The importance of haploids seems paradoxical as for breeding one would like pure lines, that is, a diploid where both sets of chromosomes bear the same traits. But this is very hard to do with conventional breeding methods. One way of tackling this problem would be to obtain doubling of the chromosomes of a haploid. These plants are aptly named dihaploids. The number of generations required to develop a pure line would thus be drastically reduced. Presently, there are some problems with this technique. Regenerated dihaploid plants either cannot be obtained in some economically important plants, or else their induction frequency is very low as in soybean and maize. Another major problem is that dihaploids often show chromosomal instability. Thus till now they have not been commercially exploited.



Plant breeders have contributed to the improvement of crops by combining desirable genes into a cultivar. In many cases the desired traits have been obtained by crossing with wild plants. The farmer's contribution too cannot be underestimated, as over the years they have selected varieties suitable to their locales. But both these methods are endangered because of the erosion of the **biological diversity**. Today destruction of forests and other natural habitats is resulting in an alarming loss of plant species. It is expected that at the present rate, one quarter (about 60,000) of plant species could be lost by the middle of the next century. Of the



Back to the future

cultivars, a huge number has been lost due to the introduction of only a few high yielding varieties. We are not only losing cultivars, but along with it we are losing the genes which might one day come in handy to introduce a particular trait into a crop especially when with the advent of biotechnology we can overcome barriers between incompatible species. We are also losing a potential source of yet undiscovered food plants and those that provide other important necessities including medicines and biochemicals. While it may not be possible to save all the species and cultivars at least a large number can be saved for the future generations through storage in germplasm banks or gene banks as they are commonly known.

The easiest and most economical way to save germplasm is of course, to save seeds of known species and varieties under conditions in which their chance of remaining viable are



high. Seed banking, however, has its problems. Some crop plants do not produce viable seeds. Some seeds are recalcitrant; these are usually the large and succulent seeds, which lack dormancy, and cannot withstand low temperatures or dehydration. Other seeds deteriorate rapidly because they are infected by pathogens. Some varieties are heterozygous, and do not produce plants with identical traits everytime. Lastly one cannot use seed banks for storage of vegetatively propagated plants. Ginger, onion, garlic and potato can no doubt be stored, but not for long periods. Besides they are

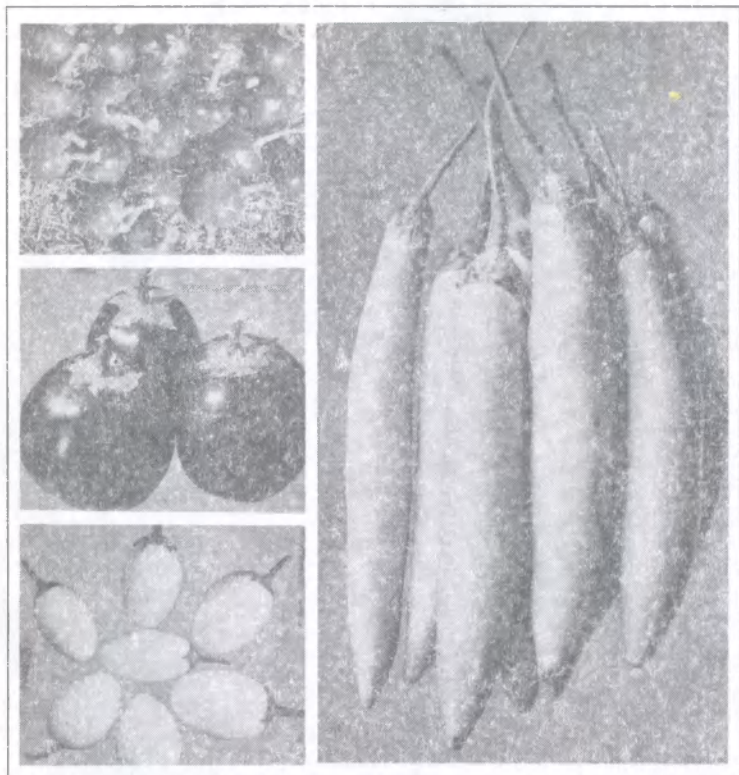
rather bulky materials and would require a great deal of space.

With the development of tissue culture techniques the problem has been somewhat solved. There are two methods of long term storage of tissue cultures — the first is by slowing down growth and the second by arresting growth by storing the tissues at very low temperatures.

Storage at very low temperatures is called cryopreservation. The only way to totally arrest growth is by reducing the temperature to that of liquid nitrogen (-196°C). However, most biological materials are prone to damage when frozen and subsequently thawed. So certain specific conditions have to be followed to obtain viable arrested cultures. Since most of the water in the plant cells is to be found in vacuoles, tissues having small vacuoles are ideal for cryopreservation. Fast growing tissues where the cells are dividing but not expanding very much are ideal as they have relatively small vacuoles. and in such tissues there is less damage due to ice crystal formation. Certain chemicals when added to the culture medium protect the tissues by preventing the formation of ice crystals. The next step is the actual freezing. One can freeze fast, in which case there is little dehydration because intracellular freezing occurs first. While in slow freezing the outer layers are frozen first, followed by dehydration of the cytoplasm. There is no hard and fast rule as to which one should be applied and therefore, the exact procedure has to be worked out for each type of material. Frozen tissues are stored in liquid nitrogen or in nitrogen gas at minus 150°C . On no account must the temperature be allowed to rise above minus 100°C during storage. When required for regeneration, tissues are thawed rapidly by dropping them into warm water or culture medium. This avoids formation of damaging ice crystals, which occur during slow thawing. When necessary, the tissues have to be carefully nursed back to normal growth after washing out the chemicals used.



Cryopreservation has been successful with a wide variety of plants, and for a range of tissues. The greatest drawback of the method is that it is not possible to recover all the frozen samples, which implies that the germplasm banks would be required to store substantial quantities of tissues. The gene banks of food crops are located at eighteen international agricultural research centres, under the Consultative Group on International Agricultural Research (CGIAR). They store over 500,000 germplasm collections of agricultural crops. In India, the National Bureau for Plant Genetic Resources



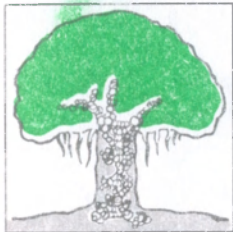
Variety is the spice of life

(NBPGR) houses the gene bank. A National Facility for Plant Tissue Culture Repository (NFPTCR) has been set up at the NBPGR. The tissue culture bank focuses on tubers, bulbs, spices, plantation crops, horticultural crops and medicinal and aromatic plants. The actual working is, however, very time consuming as a proper method has to be developed for each species and sometimes even cultivars. Such banks may prove to be the saviour of the human race when earth's natural resources are depleted and feeding of an exploding population assumes top priority.



Plant breeders keep on dreaming of making exotic crosses. The advent of techniques such as somatic hybridization and recombinant DNA technology have made it possible to translate a few such dreams into reality.

About the same time as research on plant tissue culture started, there were other groups of scientists who were studying the effect of fungal enzymes on plant cells. In 1960, E.C. Cocking reported the isolation of protoplasts using appropriate combination of enzymes that fungi use to break down the cell wall of plants. Protoplasts are contents of the plant cell without the cell wall and surrounded only by the cell

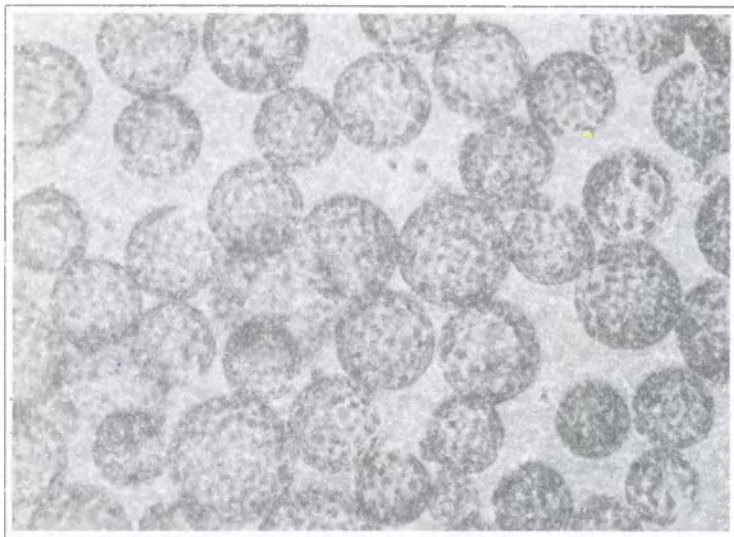


Engineering better plants

membrane. Within the next decade he so refined the process that it was possible to liberate billions of protoplasts within a few hours. Other scientists were then able to regenerate whole plants from single protoplasts.

Protoplasts can be produced from cells cultured in liquid medium, callus tissues or intact tissues such as young leaves by mechanical disruption. It is, however, preferable to produce them by treatment with the enzymes cellulose and pectinase. The latter is necessary to break up cell aggregates into individual cells and the former to remove the cell wall proper. After treatment with enzymes, the protoplast suspensions are collected and plated on to a nutrient medium. Protoplasts synthesize new cell walls in five to ten days and then initiate cell division.

Once protoplasts could be obtained, the possibility of fusion of protoplasts from different species opened up. Spon-



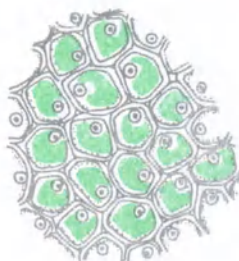
Protoplasts in culture media

taneous fusion of protoplasts does occur, especially when they are isolated from young leaves. Fusion between protoplasts of two different species can be induced by treating them with certain chemicals called 'fusogens'. Alternatively a technique called electrofusion is used. Protoplasts tend to adhere in a non-uniform electrical field. Fusion takes place when an electric shock is applied. These methods are now being used to produce 'somatic hybrids' by fusing protoplasts from two different species.

When protoplast fusion occurs between two species the first product is a 'heterokaryon', which contains both the cytoplasm and the two nuclei. If the two nuclei subsequently fuse the cell is known as a 'synkaryon'. Although fusion brings together the chloroplast and mitochondria from two species, they do not continue to exist together, and ultimately those from one plant predominate. In many cases of somatic hybridization there is also cellular incompatibility in that it is impossible for the two nuclei to exist intact in the same



Dissolving cell walls of leaves



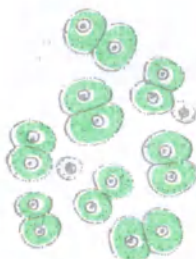
Protoplast made to shrink and become spherical



Isolated protoplasts transferred to a culture medium



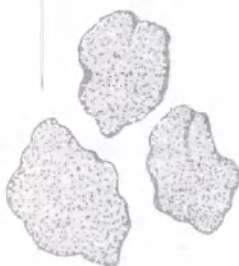
Thriving protoplasts



Microcallus



Microcallus transferred to second culture medium



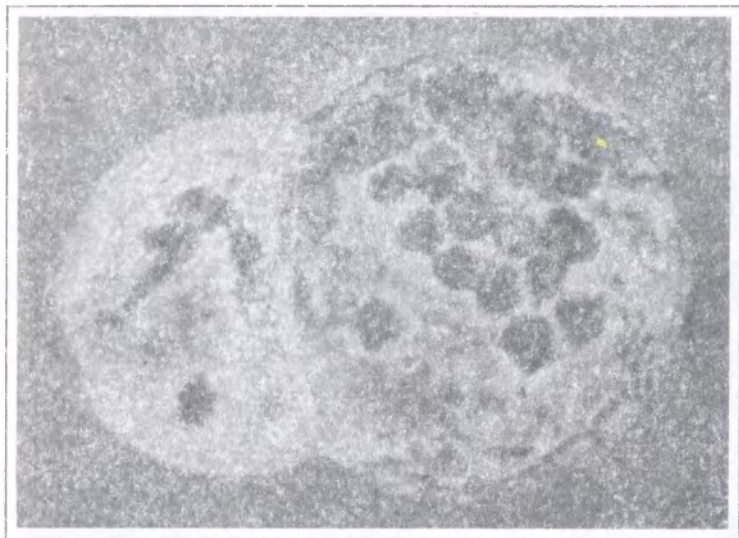
Callus



Differentiation



Plantlet



Fusion of protoplasts

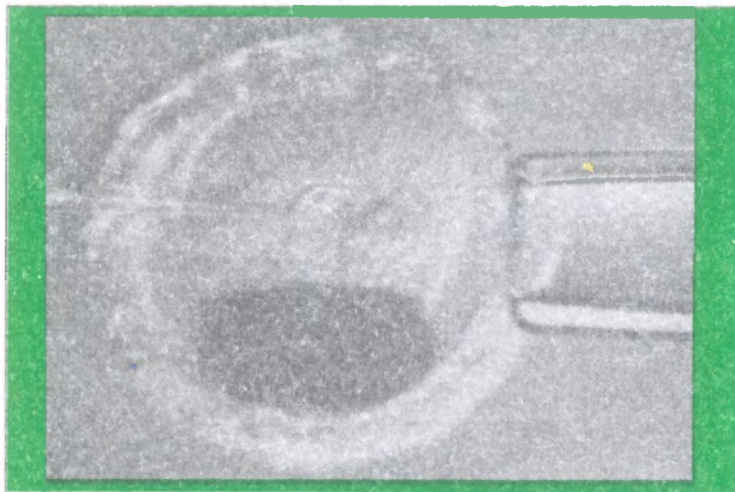
cytoplasm. So what happens is that some of the chromosomes are eliminated. In extreme cases one of the nuclei is lost. Thus one may get cells having nucleus from one parent and cytoplasmic organelles from the other. These cells are known as 'cytoplasmic hybrids' or 'cybrids'.

Chloroplasts and mitochondria possess DNA of their own which code for some of the important agricultural traits. In somatic hybrids the nuclear and cytoplasmic genomes recombine to produce a wide range of gene combinations not possible through conventional breeding techniques. The somatic hybrids can then be grown *in vitro* to produce callus tissue, from which plants can be regenerated. In some cases, the protoplasts from one parent is exposed to *gamma* rays. The resultant hybrid is called an 'asymmetric hybrid'. These hybrids possess only a small part of the genome, the rest being inactivated by *gamma* radiation. Their use allows transfer of a small fraction of the genome in a single hybridization step.

Somatic hybridization is attempted in cases of species which are incompatible to cross breeding. However, only limited success has been obtained because of certain inherent drawbacks. The first is the inability to regenerate plants in a large number of species. Second is the infertility of some somatic hybrids, and last but not the least, it is hard to obtain the precise genetic recombination essential for improvement of the crop.

Though of little commercial value, the most well known somatic hybrid is the 'pomato' obtained by the fusion of the cells from tomato and potato. Some useful somatic hybrids are cytoplasmic male sterile rice, oil seed rape or canola resistant to the fungus *Phoma lingam* and potato varieties resistant to certain virus diseases. In India, protoplast fusion and regeneration of hybrid plants have been achieved, between Indian mustard and its wild relatives. The purpose of these experiments was to introduce genes for stress resistance from the wild species into the cultivated ones.

Another approach which has been widely explored is the introduction of foreign DNA into plant cells by various physical methods. There are a number of ways by which plant protoplasts can be induced to take up exogenous naked DNA or DNA without the coating of proteins. One of the ways is by calcium phosphate mediated DNA uptake in which DNA and calcium phosphate from a solution is taken up by protoplasts. The major drawback of this method is that very few of the cells actually take up DNA. An alternative is to inject the DNA directly into the protoplast. While microinjection is possible, the process has not been much used as it often damages the protoplast. DNA encapsulated in artificial membrane enclosed vesicles are known as liposomes and these are another way of introducing exogenous DNA into protoplasts. The liposomes fuse with the plasma membrane and discharge their contents into the protoplast. Electroporation has also been used to introduce foreign DNA into protoplasts. The process involves passing of short pulses of electric current

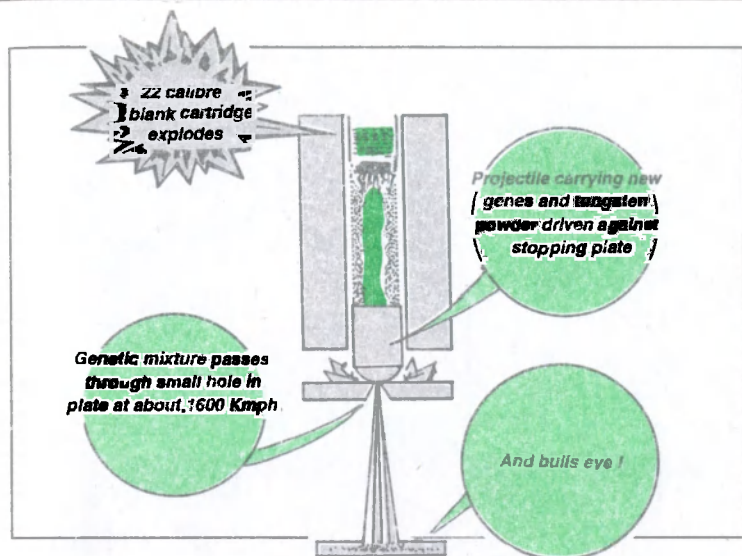


Microinjection

which makes the cell membrane temporarily permeable to large molecules such as DNA, by opening up the pores of the membrane.

All the above methods involve the handling of protoplasts, which in itself is a tricky proposition requiring a high degree of expertise and as a result even though it has been demonstrated that these methods are possible they have not been seriously exploited.

The current approach is to try and introduce the DNA into intact cells rather than protoplasts. This has been achieved in two ways. The first is by shooting the DNA into plant cells using high velocity microprojectiles. The process is called 'biolistics'. The DNA is coated onto spherical tungsten particles which are literally shot into the cells using a gunpowder charge. This remarkable technique does not require cell cultures or pretreatment of recipient tissue and bypasses the problems of handling and regenerating protoplasts. However, the equipment required for biolistics is highly expensive.



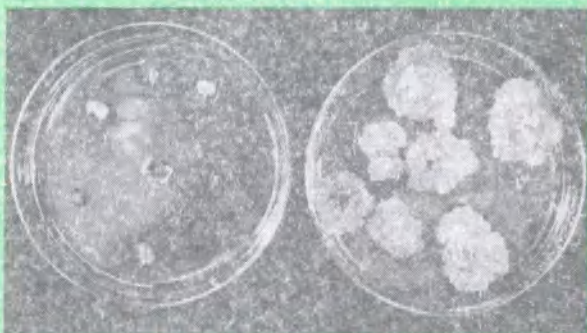
Firing the gene gun

The latest method being patented by the British biotechnology firm Zeneca, involves the use of silicon carbide particles. The silicon carbide process is extremely simple and does not require any special apparatus. Tiny silicon carbide crystals in a suitable medium with tiny loops of genetic material containing DNA to be inserted are all that is needed. Then the recipient plant cells are introduced and swished around the mixture for a minute. The hard sharp crystals punch holes in the cell membrane through which the foreign DNA can enter. Apparently the holes are repaired very fast by the cells allowing the DNA to be retained inside. Being a very recent method its applicability on a practical scale is yet to be demonstrated.

Cereals (wheat, barley, maize, rice, sorghum and millets) together provide fifty-two per cent of the food consumed by human beings. So far as genetic engineering is concerned they have proved rather recalcitrant. It is only in the last few years that it has been possible to introduce foreign genes into *Japonica* and *Indica* varieties of rice, maize, wheat and



Embryogenic cell suspension (ECS)



Selecting transgenics

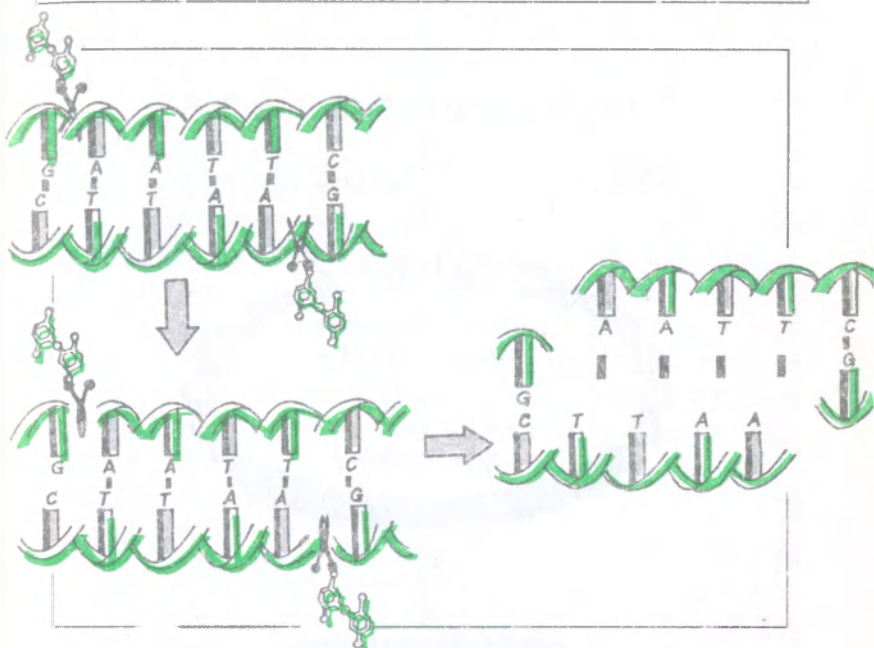


Differentiation



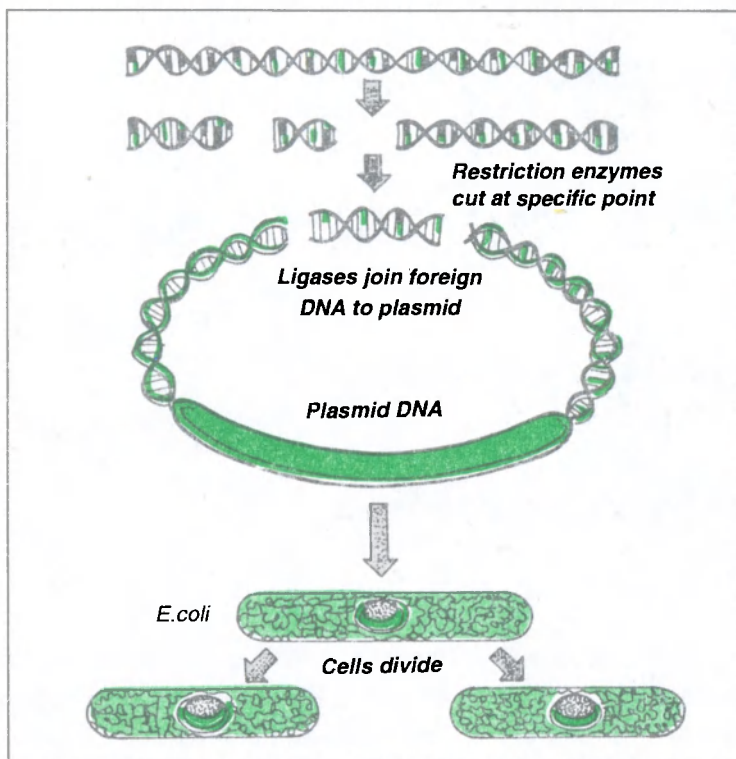
Transgenic plants

Making transgenic rice



Chopping up DNA

sorghum. The scientists therefore looked for a more biological approach to the introduction of foreign DNA into plant cells. Enzymes which were to form key tools in recombinant DNA technology, which forms the base for genetic engineering have long been discovered. The first of these enzymes was DNA polymerase discovered by the American scientist Arthur Kornberg in 1967. The enzyme is responsible for joining nucleotides into a DNA strand. The new strand can only be formed on a template strand of DNA to which it is complementary. Kornberg and his co-workers were able to synthesize biologically active DNA using this enzyme and virus DNA as template. The enzyme can thus be used *in vitro* to assemble specific DNA sequences and/or genes. The second important enzyme was DNA ligase discovered in 1967. This enzyme can weld DNA molecules together, and is particularly useful for repairing breaks in DNA strands.



Doctoring the plasmid

In 1968, the Swiss scientist Werner Arber discovered a group of enzymes called restriction endonucleases. By 1971, Daniel Nathans and Hamilton Smith were able to isolate a number of these enzymes. Restriction enzymes as they are commonly called, recognise short specific nucleotide sequences in DNA molecules and cut specific points within these sequences. The enzyme cuts the phosphodiester bonds of both the DNA strands in a molecule. This generates a set of double stranded DNA fragments with single stranded ends called "sticky ends". Although not really sticky, these ends can form base pairs with DNA from another source cut by the same enzyme. The gaps in the strand are then joined

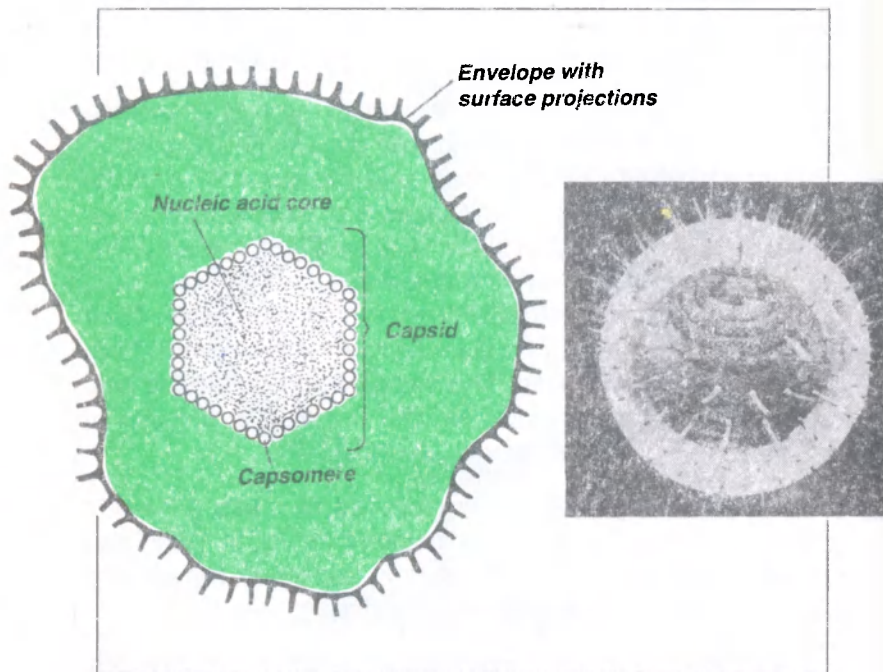
by DNA ligase. To date hundreds of restriction enzymes and about a hundred specific recognition sites have been discovered.

Isolation of the various enzymes paved the way for routine cutting and pasting of DNA in genetic engineering. By 1972-73, the first hybrid DNA molecule was produced by artificial means. About the same time two American scientists, Stanley Cohen and Herbert Boyer were able to splice foreign DNA into a plasmid. A plasmid is a small circular DNA molecule found in bacteria in addition to the main chromosome. They then introduced it back into *Escherichia coli* a common bacteria, which then multiplied. This experiment opened the way for cloning genes into bacteria, and ushered in the era of genetic engineering.

By 1977, *E.coli* could be engineered to make a human hormone, somatostatin. Human insulin followed in 1978, and by 1980, bacteria were commercially exploited to give the world first genetically engineered product.

It was felt that advances in recombinant DNA technology could be fruitfully applied to plants if proper vectors such as the plasmids which introduce foreign genes into bacteria could be obtained. This would enable transfer of genes between incompatible plants and between plants and other organisms. The sort of genetic engineering that is available for bacteria would be a boon to plant breeders, since it would not only be faster than conventional methods of obtaining hybrids, but it would also allow access to a larger gene pool including genes from bacteria and animals.

At first glance, viruses seem to be a suitable vector for genetic engineering of plants. They consist of a nucleic acid core surrounded by a coat of protein. Not only can viruses infect plants but even their purified nucleic acid is infectious. However, to act as a suitable vector viruses have to fulfil certain conditions. They must be able to move from cell to cell. Also since the addition of extra nucleic acid during



Virus — at the borderline of life

genetic engineering may make the viral DNA too large to fit into the protein coat, the viral nucleic acid must be able to move from cell to cell without its coat. The modified viral nucleic acid should not cause disease symptoms. The virus should also have a wide range of hosts in order to become a practical tool. However, since genetic engineering of plants involves manipulation of DNA, only DNA viruses would be suitable to act as vectors.

Only two groups of plant viruses, the caulimoviruses and the geminiviruses have DNA genomes. Among the caulimoviruses, the cauliflower mosaic virus (CaMV) has been widely explored as a vector. However, CaMV has two major drawbacks. The first is the small size of DNA inserts which can be used with this vector and the second is its very limited

host range. Viruses in general have other limitation as vectors. They are not easily transmitted through pollen or seed, and thus offer problems in breeding of new varieties as their distribution throughout the host is not uniform and also because these vectors have a high degree of genetic instability.

The answer to the search for a suitable vector for plant genetic engineering came from quite an unexpected source. The vector was the tumour-inducing plasmid (Ti) of *Agrobacterium tumefaciens*. The circular DNA contains a section called T- DNA which is actually transferred to the plant. Normally this section contains the *onc* genes whose products are responsible for tumour formation. In addition there is a *nos* gene responsible for the synthesis of nopaline, an unusual amino acid. The T-DNA sector is bordered by DNA that has 25-base pair direct repeats. These sequences are essential for its transfer. Any DNA placed between the two borders becomes T- DNA. The circular DNA also contains another gene *vir*, which is also essential for T-DNA transfer to the host.

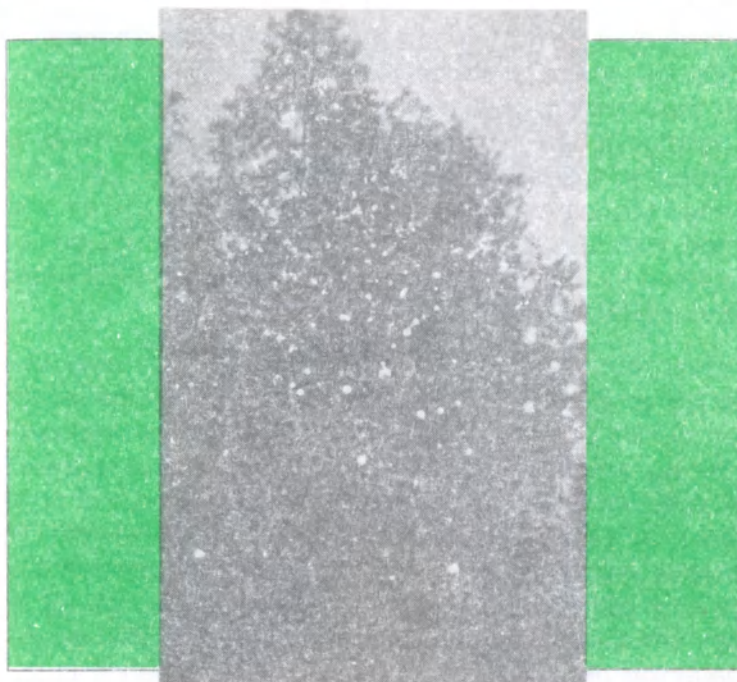
The first step in modifying the vector is to 'disarm' the Ti plasmid, that is get rid of its tumour producing activity. Most of the T-DNA sector is snipped out and useful genes put in its place. All other genetic functions of the vector remains unaltered, so that when the plasmid is introduced into the plant cell host, the useful gene is integrated into its DNA, and expressed in subsequent generations. In order for an alien gene to be expressed in plant cells it is essential that it contains a promoter region, a sequence of DNA immediately preceding the sector which will be translated, and which is recognised by the RNA polymerase of the host.

Since the Ti plasmid is extremely large, a system of intermediate size vectors have been developed by snipping out some unrequired portions of the DNA. These vectors are able to reproduce both in *E. coli* and in *A. tumefaciens*. The required genes are manipulated into the vector *in vitro*. They are then introduced into *E. coli* and then transferred to *A. tumefaciens*. The genetically modified *A. tumefaciens* then



interacts with the plant cells, which on further development gives rise to the 'transgenic' plants.

The integration of alien genes into plant chromosomes during genetic modification occurs at very low frequencies. May be only one in a thousand or even million cells will carry the gene stably. In order to identify these cells, 'selective marker' genes are incorporated into the vector by joining them to the gene of interest. Only cells that will contain the marker genes will survive the selective pressure imposed in the laboratory on the cells undergoing modification. Plants regenerated from these cells will contain the selective marker joined to the agronomic trait.



Lighting up

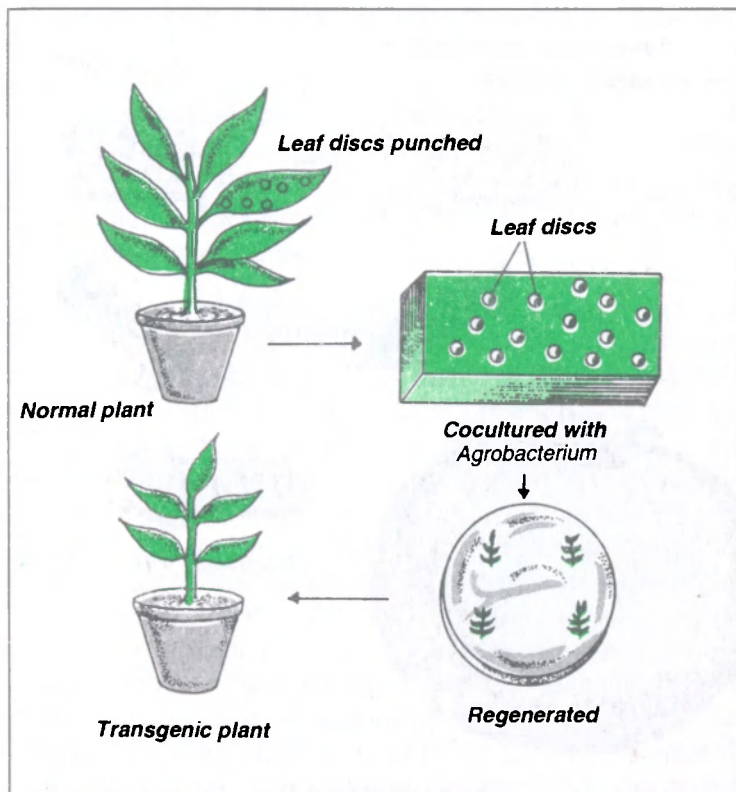
Two main classes of selective markers are in use. The first encodes proteins that confer resistance to antibiotics such as kanamycin, gentamycin and teleomycin. The second confers tolerance to herbicides such as phosphinotricin, sulphonyl ureas and 2,4-D. The commonest marker is the gene encoding neomycin phosphotransferase II (NPT II) which inactivates kanamycin G418, and neomycin. Over thirty transgenic plants which contain NPT II as marker have been field tested. The antibiotics and herbicides are only used in the initial tissue culture stage to identify the transformed cells. The transgenic plants contain neither the antibiotic nor the herbicide, but they do contain the marker gene.

'Scoreable marker' genes also called 'reporter' genes are those which can be used to identify and track the genetic modifications. However, unlike selectable markers the score-

able markers do not facilitate the survival of transformed cells under experimental conditions; rather they tag and identify the transformed cells from among the host of non-transformed cells. The two most important scoreable markers are B-glucuronidase (GUS) and luciferase (LUC) genes. Transformed tissues expressing GUS gene turn blue when incubated with certain chemicals. Presence of LUC gene is identified by the production of light when they are incubated with luciferin.

Even though the vector is carried by *A. tumefaciens* which has the ability to directly enter the plant through the wounds and modify at least a part of it, this is not the method of inducing transgenic plants. Instead, leaf discs of species to be transformed are exposed overnight under sterile conditions to *A. tumefaciens* carrying the modified Ti plasmid. The bacteria infects the cut edge of the discs. The treated discs are next placed on a shoot-inducing liquid medium for two days and then transferred to a shoot inducing solid medium containing antibiotics which inhibit shoot formation in non-transformed cells. On the other hand transformed cells break down the antibiotics and therefore produce shoots. After about twenty days, the callus with its shoot is excised and transferred to a antibiotic containing root inducing solid medium. Four to six weeks later when the roots have appeared, the plantlets are transferred to the soil.

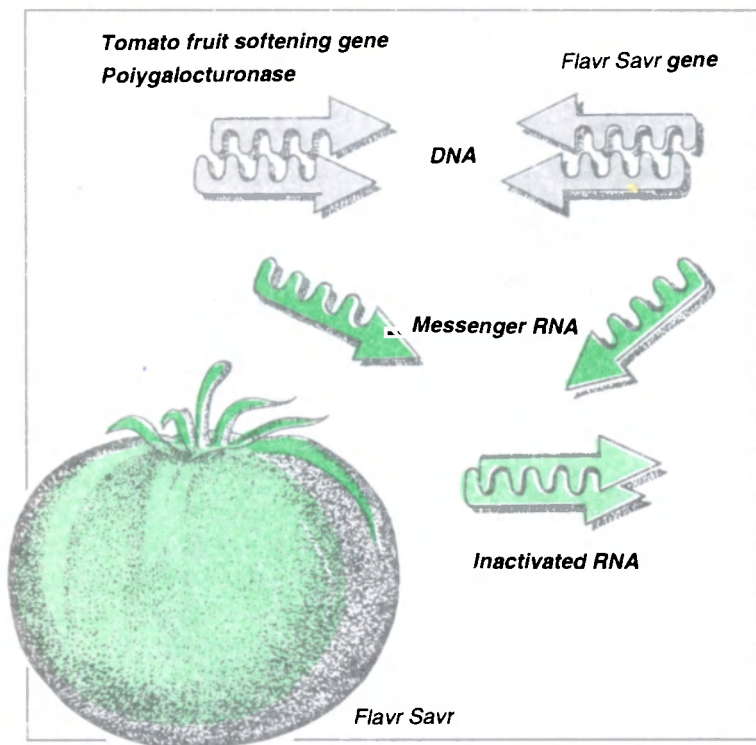
The first transgenic plant engineered using the Ti plasmid as vector was produced a decade ago. Since then improved agronomic traits have been introduced into almost fifty crop plants. Sources of the introduced genes vary from those borrowed from other species of plants, bacteria, animals, viruses and extends to synthesized genes. Disease, pest and stress resistance and herbicide tolerance have been the major focus. Tomato growers and consumers have been one of the first to profit from it. Tomatoes once plucked get mushy very quickly, and lose flavour. Since the fruit is easily perishable, growers pick the tomatoes while still green and hard.



Tissue culture technique

These are ripened by treatment with ethylene gas which is also involved in the natural ripening of fruits. While such tomatoes look good, they are not as flavourful as those that ripen on the vine. Growers are not concerned with flavour while to the consumer it is a major criterion.

Scientists sided with the consumers and at Calgene in California, researchers engineered a tomato variety called *Flavr Savr*, which can be left to ripen in the field. Once picked it has a shelf life of two weeks, double that of the current commercial varieties.



The secret of Flavr Savr

Flavr Savr has been engineered in an ingenious way by the inclusion of a gene which produces antisense RNA. Antisense RNA is exactly what its name implies and owes its existence to the helical nature of DNA. A stretch of DNA along one of the double strands codes for a particular protein. In order to manufacture this protein the DNA is first transcribed into a messenger RNA (mRNA), which unlike DNA is a single stranded molecule. This process is facilitated by an enzyme RNA polymerase using the single-stranded DNA as a template. The mRNA then passes into the cytoplasm, where it is translated into proteins. The complementary strand of DNA

is, however, not transcribed. If one can clone this complementary strand and reintroduce into the plant in the proper orientation then it would produce an antisense RNA which would block the action of mRNA. How antisense RNA works is still not well understood but whatever the mechanism, antisense RNA does inhibit its complementary mRNA. This character of antisense RNA was utilised by the researchers at Calgene.

Tomatoes become mushy because they produce a softening enzyme called polygalacturonase (PG), which attacks the pectin in the cell walls and causes the cells to come apart. All other changes associated with tomato ripening such as flavour and colour development, are not affected by this enzyme. The Calgene scientists were successful in cloning the complementary DNA of tomato PG. They then inserted it into tomatoes in the antisense orientation. These transgenic tomatoes exhibited decreases in PG level up to 99 percent, thereby increasing the shelf life. What is the most interesting thing about using antisense RNA is that one is not introducing a foreign gene into the plant except, of course for the NPT II marker. The *Flavr Savr* tomatoes have been cleared by the Food and Drug Administration of the U.S.A. for release in the market. It is the first genetically engineered food on the market.

While Calgene has already started to market its product, other groups of researchers are continuing their investigations taking a different approach to the problem. They are focusing on the ethylene biosynthesis pathway. By using antisense RNA to produce blocks in ethylene synthesis, American researchers could produce transgenic plants with a delayed onset of ripening.

Approaching the problem in a still different manner is DNA Plant Technology, New Jersey, USA. They claim to have developed a technology which they call 'transwitch'. Here the gene of interest is duplicated and an exact copy is inserted

back in the plant. According to them this "inhibits the translation of RNA into protein, but how that happens is not completely well characterised". It seems clear that exciting days lie ahead for plant genetic engineering.



Crop plants especially the high yielding varieties, are particularly prone to diseases and pests. It is estimated that Rs 6000 crores worth of agricultural produce are destroyed by pests annually. Weeds take up 30 to 50 per cent of the total nutrients supplied to the crops, and 20 to 40 per cent of the soil moisture. With expanding population productive land is at a premium. Therefore it is imperative to control pests, weeds and disease in order to maximise yield.

Plant protection chemicals have been developed to control pests, but the problem not only continues but is increasing



Good riddance

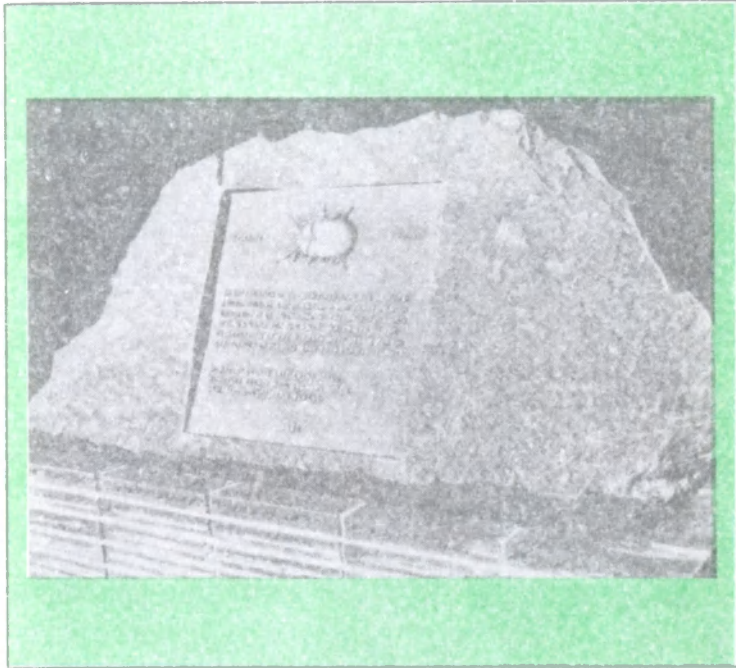
day by day. Continuous use of pesticides has led to the development of resistant strains of insects. Chemical pesticides are indiscriminate in action and also kill beneficial insects which help in pollination and control of harmful insects. Pesticides are harmful also to man and animals. They are not easily degraded in the soil, and tend to accumulate in the food chains, leading to amplification of their effects. Diseases in crops are caused by bacteria, fungi and viruses. There are some chemicals which are useful against certain bacteria and fungi, but none against viruses. The only method available till recently was to breed disease resistant plants by conventional methods. This is very time consuming, often taking years, while in the mean time crops continue to be devastated.

Biotechnology has taken over where chemistry has failed. In recent years, control of disease by biological agents (bio-control) has become a major field of endeavour. Bacu-



loviruses are one such group of biocontrol agents. These viruses are highly invertebrate-specific and safe for vertebrates such as poultry and fish. Beneficial insects such as honey bees or parasitic insects that prey on pests are not affected by them. Another desirable property of many of the baculoviruses is they are enclosed in large crystals which protect the viruses from degradation in the open environment.

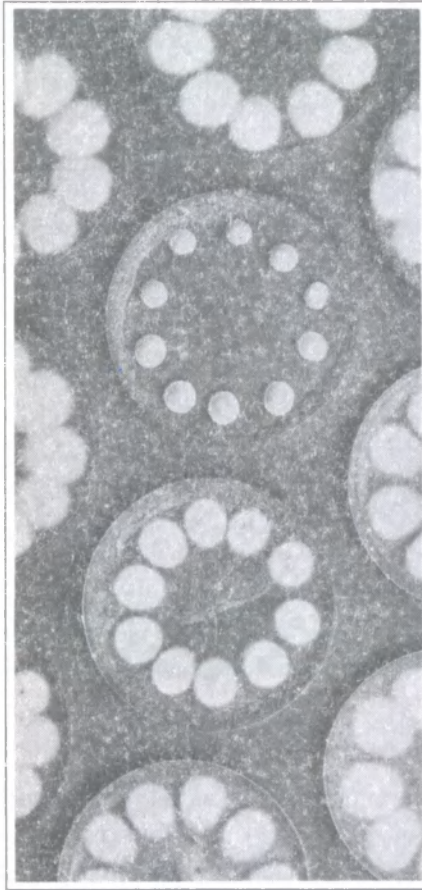
Baculoviruses infect several species of butterflies and moths and most of the species affected are pests of important crops. Some members infect other insects such as ants, bees, wasps, flies, gnats, midges, beetles and caddis flies. However, the host range of a particular species of baculovirus is usually restricted to only a few species within a particular genera of insects. Because of their specificity several baculoviruses have been registered as commercial pesticides. Baculoviruses only grow and multiply within the host cell.



Thanksgiving monument to a beetle that helped
Californian ranchers

Unlike bacteria they cannot be cultured *in vitro* in a synthetic medium. Thus technologies have been developed for their production on a commercial scale. The first viral insecticide to be developed was from *Heliothis zea* in 1961, and was marketed in 1975 by Sandoz under the trade name ELCAR.

In addition to the viruses, bacteria also act as potent biocontrol agents. *Bacillus thuringiensis* (B t) is commercially available as Thuricide HP, Biotrol BTB 25W and Thuricide dust. Different strains are used for different species. This bacterium is highly suited to integrated pest management programmes in which chemical control is used along with biocontrols. Besides producing bacterial formulations it has been possible to produce the crystalline toxin commercially.



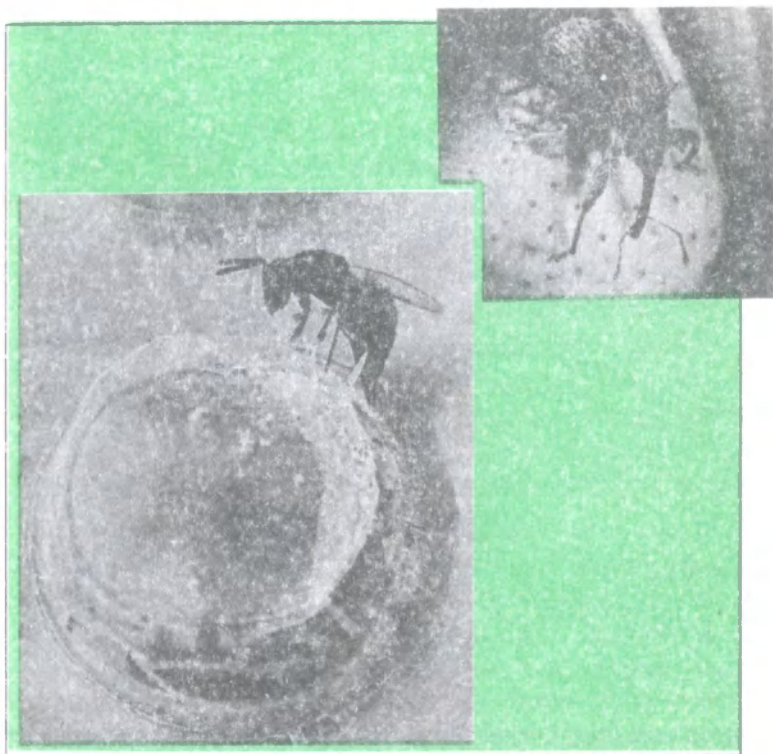
Verticillium lecanii pellets

Some scientists have even gone a step further. They have been able to produce genetically engineered tobacco plants which make the B t toxin. This endogenous production of the B t toxin does not harm the plants but confers on it the ability to withstand insect attack.

Fungi such as *Beauveria*, *Verticillium lecanii* and *Hirsutella thompsonii* have been found to be effective against several crop pests. But unlike the bacterial and viral agents the fungi are not commercially available. These fungi are not harmful to man.

Nematodes which are tiny worms found in the soil can be both beneficial as well as pathogenic. Some nematodes like *Steinernema* and

Heterorhabditis have bacteria in their alimentary tracts. When infected by nematodes, the bacteria enter the insect system where they multiply and kill the hosts within 48 hours. Nematodes are particularly suitable bio-control agents for areas where applications of chemicals may highly endanger the environment. They are suitable for use near water systems, or in the vicinity of livestock.



Parasitic fly laying egg on boll-worm larva.
Adult bollworm (inset)

Insects can also control insect pests by either being parasitic on them or by preying on them. Scientists of the International Rice Research Institute in the Philippines say that there are about 800 species of friendly insects in a typical Philippines rice field. They outnumber pests by three to five times. These include beetles, wasps, bugs and praying mantis. Small predatory bugs that inhabit the surface of paddy waters, attack all plant hoppers and leaf hoppers. Crickets feed on the egg mass of the striped stemborer, while young larvae are juicy food for a predatory beetle. Nature provides all these controls, however, when chemical insecticides are applied, the first spraying kills off the beneficial insects more than

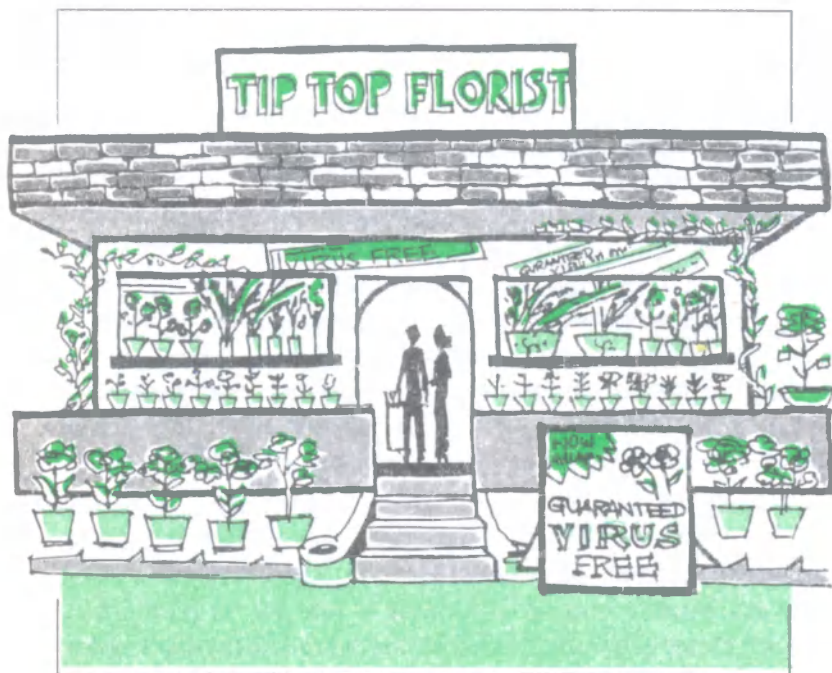


pests and the sturdier pests are then free to attack the crops. A species of wasps, *Trichogamma* is particularly effective as a parasite. They lay their eggs on the eggs of the pest, and after the larvae hatching feed on the larvae of the pest *Trichogamma* are commercially produced in insectaries. One company produces 50-100 million wasps per day. This company supplies three species, *T. platneri*, *T. minutum* and *T. pretiosum* for control of caterpillar in cotton, corn, apple, spruce and avocado. In India pilot scale trials with *T. chilonis* has been successful in controlling sugarcane internode borer.

Whenever one thinks of predators for insects, the first thing that comes to mind are spiders. Wolf spiders, jumping spiders and crab spiders are the most efficient. They do not entangle their prey in webs, but rather they pounce on them and paralyse them with their poison fangs. Wolf spiders live on the ground, jumping spiders on plants and crab spiders in

flowers. In an experiment reported in China, spiders killed up to 90 per cent of the harmful insects in cotton fields. Scientists created a proper environment for nurturing the spiders by digging shallow pits and lining them with straw, for spiders to shelter from the cold. With this small investment some provinces saved up to 60 per cent of the pesticide commonly used. A wolf spider can eat up ten to twenty brown plant hoppers which is Asia's most destructive rice pest, and stemborers everyday. The latter are almost immune to pesticides as they spend most of their life inside the plant. Spiders however are able to sense and pry out these insects by squeezing into the stems. A hectare of arable land may harbour as many as 1.2 million spiders. Imagine having a million helping hands to pick out and destroy pests. What better friends do farmers need!

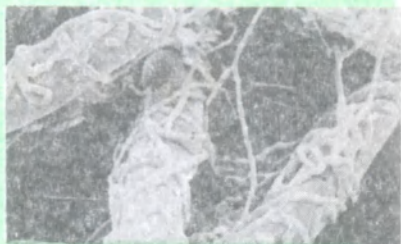
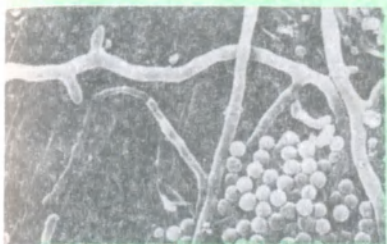
While there are many ways of biocontrolling insects, viruses, bacteria and fungi are more recalcitrant. Viruses are usually transmitted through sucking insects such as aphids or are seed borne. In a number of species of plants it has been observed that growing points have low concentration of viruses. The growing tip of a plant is metabolically very active. Being undifferentiated, it is also free from conducting tissues which are the normal pathway of movement of viruses through the plant. In certain crops and ornamental plants it is economical to use the tissue culture techniques for obtaining virus free somatic embryos. This is done by regenerating virusfree explants of tips. Tips are excised from young, rapidly growing presterilized seedlings or from shoots obtained from tissue culture. Numerous factors influence the ability to eliminate virus by this method. Both location and the size of the bud to be excised are important. The best is to culture the smallest explant, however, too small explants do not produce roots. The optimum size has to be established through trial and error. Further, heat treatment inactivates virus without affecting the plant material. Using meristem culture it has been possible to eliminate mottle virus from strawberry, potato virus X from potato, mosaic virus from cassava and



cauliflower mosaic virus from cauliflower. Virus free plants give higher yields, sometimes by as much as 60 per cent.

Plants regenerated from callus tissue, may also be free from viral infection. Callus tissue like cells from the growing tip have a high division rate and incomplete vascular system. Virus free plants have been obtained from callus in tobacco, geranium, gladiolus and potato. It is also possible to use uninfected leaf tissue to generate virus free plants.

To control pathogenic bacteria and fungi, non-pathogenic life forms are used. For instance the fungus *Trichoderma* has been successfully used to control damping off disease in peas, tomato and tobacco caused by *Pythium* and *Rhizoctonia solani* and root rot of groundnut. *Gliocladium virens* can be used to control diseases caused by *Pythium*, *Sclerotium* and *Rhizoctonia*. The bacteria *Pseudomonas fluorescens*



Spores of *Trichoderma* begin to send threads around a lettuce leaf



These coil around *Rhizoctonia*, burrow into it and kill it

Fungal friends

and *P. putida* are effective in controlling soft rot of potato caused by *Erwinia carotovora*. These micro-organisms have to be added to the soil under appropriate conditions to control disease. When fungi is used as a biocontrol agent no antifungal chemical agents are added to the soil as it affects both the beneficial as well as the pathogenic fungi.

Whenever a pesticide or a herbicide is applied to get rid of a noxious organism, a few organisms always manage to survive. One way to develop herbicide resistant crop plants would be to expose the plants to the herbicide and select the ones that survive for further screening. Under standard breeding practices it could take years before a strain could be stabilised for commercial release. Biotechnology however has provided tools for obtaining such plants fairly quickly. Two methods have received attention — one is screening of plant cell cultures for herbicides resistant cells and then trying to

regenerate plants from these cells. Alternatively, transgenic plants could be developed which would have herbicide resistance.

A wide variety of herbicide resistant crops have now been developed. What is more interesting is that a certain proportion of these plants are also resistant to other herbicides with an allied chemical structure. For example, some paraquat resistant soybean cell lines and tobacco cell lines are also resistant to the related diquat, and two of five tobacco cell lines resistant to amitrol are also resistant to glyphosate.

The most exciting breakthrough in breeding of herbicide resistant plants is the transfer of genes which disrupt the



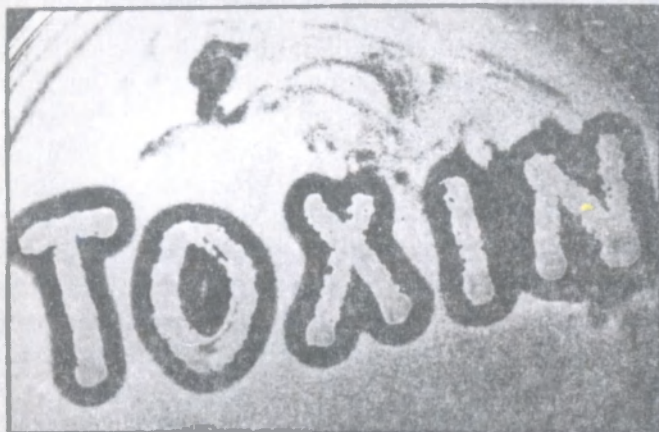
More power to the crop plants!

pathway of herbicide action. For this an understanding of the mode of action of the herbicide is essential and it is now known that many of these act to inhibit biosynthesis of amino acids which are the building blocks of proteins.

Glyphosate is a broad spectrum herbicide normally used to kill the weeds before the crops is sown. However, if one could develop glyphosate resistant crops then this chemical could also be used during their raising. In the first step glyphosate resistant *E.coli* were produced. Transgenic plants were produced using the resistance genes from the bacteria. So given a particular concentration of the herbicide, the enzymes of the normal organism totally bind to glyphosate and amino acid synthesis stops. In the resistant plant enough additional enzymes is produced for the normal pathway of synthesis to continue even after glyphosate binding.

Sulphonylureas are effective broad spectrum herbicides used on cereals. They kill most plants and residues left in the soil damage crops sown after cereals. In order to engineer resistance to the herbicide, *Salmonella typhimurium* strains resistant to chlorsulfuron was first isolated. The gene responsible for this resistance was named *ilvG*. The *ilvG* gene when transferred to tobacco rendered it resistant to the sulphonylurea herbicides.

Another approach to the problem is to introduce a gene encoding, an enzyme that degrades or detoxifies the herbicide. Basta, manufactured by Hoechst of Germany is a herbicide which is quite popular with the farmers because of its ability to kill any weed, but it also kills all crop plants. So it can only be applied before the crop germinates. Basta acts by 'choking' the plants on the ammonia they produce during photosynthesis. Chloroplasts produce ammonia which is rendered harmless by an enzyme called glutamine synthase which helps produce glutamine. During the reaction glutamate binds to the enzyme. Now phosphinothricine, the active ingredient of Basta resembles glutamate in structure. It competes with glutamate for the binding site on the enzyme.



Spelling out the appropriate word on a culture dish, patches of the yeast *Pichea acaciae* have inhibited the growth of a competing yeast (background) by releasing a cytotoxic protein.

Competitive inhibition

Phosphinothricine, however, does not react with ammonia. Thus when the enzyme is blocked by the herbicide, ammonia accumulates in the chloroplasts. This damages the chloroplasts as well as cells, leading to the death of the plant.

Scientists at Hoechst's laboratories in Germany have isolated a bacteria from the soil which contains an enzyme — phosphinothricine acetyl transferase (PAT) which deactivates phosphinothricine. They have managed to transfer the PAT gene into the Ti plasmid of *Agrobacterium tumefaciens*, and

used it as a vector to introduce into crop plants. The PAT gene is expressed in all the tissues, including leaves and stem of the transgenic crop. The trait is also stably inherited.

Resistance to two other groups of herbicides, gluphosinate and bromoxyinal, has been similarly achieved by introducing genes encoding bacterial enzymes that inactivate them.

Another group of German scientists have found a way of protecting tobacco plants from weeds and at the same time supplying them with nitrogen. Cynamide solution can be used to kill weeds in a tobacco field. Even if resistant strains are used a certain amount of damage accrues to the crop. The researchers have been successful in introducing a gene from the soil fungus *Myrothecium verrucaria*, which converts cynamide into urea. This enzyme is most active in the leaves which are most vulnerable to herbicide sprays. The transgenic tobacco thrives when sprayed with cynamide, not only because there is no competition from weeds, but also because the plants own urease enzyme converts the urea into ammonium salts, which is then used by the plants as a source of nitrogen. The genetic engineering for cynamide tolerance is expected to be exploited for other crop plants also.

While the high-tech approach of genetic engineering is being widely used to produce herbicide resistant crops, simple systems such as bioherbicides are not being ignored. Fungi, bacteria and virus have great potential for use as bioherbicides. The strategy involves releasing annually massive amounts of biological agents against specific weeds. Fungal pathogens are virulent, host specific and genetically stable. Many mycoherbicides have been produced on a large scale, field tested and registered for commercial use. *Phytophthora palmivora* a soil fungus to be marketed under the trade name De Vine for control of *Morrenia odorata* and *Colletotrichum gloeosporoides* fungus marketed as Collego for control of *Aeschymenae virginica* (L) have excited scientists by their potential power. Another approach to the production of bioherbicides is the utilization of plant and microbial

toxins. The bacteria and fungi are grown in fermenters in liquid medium, and the broths are tested for active compounds. Most of the transgenic crops field tested so far is for induced resistance to pest and diseases, and herbicide and stress tolerance. Their introduction as regular crops is being hotly debated.



Genetic engineering has always had its detractors, those who strongly object to “man playing God” and who would like to see all genetic engineering experiments stopped. The majority, however, while still not sure of what sort of problems genetically engineered organisms can cause when released in the environment, are keen to promote the scientific efforts because they see a better future through recombinant DNA technology. There are stringent rules and guidelines laid down for production, handling and commercial exploitation of genetically engineered microorganisms in most developed countries of the world. However, no such



How safe is safe?

single regulatory body exists for genetically engineered plants as yet.

In May 1986, Agracetus, a U.S. based biotechnology firm, conducted the first field trials of a genetically engineered tobacco. By 1987, many more transgenic plants having traits of agronomic importance had been created. These warranted large scale field trials before release to the farmer. By the end of 1992, more than forty such food and fibre crops were described, and about 600 more were under trial in twenty countries around the world. Since most the transgenic plants were developed in the U.S.A. current regulations that govern the release of transgenic plants in America deserve scrutiny. The U.S. Department of agriculture's Animal and Plant Health Inspection Service (USDA-APHIS) whose major job was to regulate introduction of organisms that might be harmful to plants, is also entrusted to monitor release of genetically engineered plants. They decided to do this on the basis of the



scientific protocol used to create it. The only thing of interest to them was whether the new varieties contained genetic material from a plant pest. The premise being that plants developed from material from pathogenic organisms must be evaluated to assure it does not pose a risk to other plants. This line of reasoning is also extended to cover situations where



pathogens have been used to effect plant transformation. Thus it is the protocol and not the product which is being evaluated by USDA-APHIS. The other regulating body is the Food and Drug Administration (FDA). In 1992, they ruled that transgenic plants which do not evince characteristics that raise food safety questions will be subject to usual levels of government scrutiny, just like foods created by other methods.

Bioengineered plants have been in the news lately the *Flavr Savr* tomato which has raised the hackles of the anti-bioengineered food group led by Jeremy Rifkin has been cleared by USDA in 1992, because it poses no environmental risk. In 1994, FDA gave this transgenic tomato the green signal saying that it is satisfied that the antibiotic resistance selective marker will not poison consumers or trigger allergies, or interfere with people taking antibiotics or add to the spread of antibiotic resistant pathogens.

The incorporation of antibiotic resistance selective marker genes particularly. NPT II which has been incorporated into



over thirty of the transgenic crops has raised quite a few questions such as, Is NPT II protein toxic to human beings? Will NPT II in any way hamper the efficiency of kanamycin and neomycin taken orally by human beings? Can the NPT II gene be transferred from plant to pathogenic bacteria thus making them antibiotic resistant? Will the spread of NPT II gene from transgenic plants cause environmental damage?

That NPT II is not harmful to mammals, including human beings, is well documented. The gene is routinely used in transformation of mammalian cell cultures and shows no harmful effect under these circumstances. No deleterious effect was seen when *Flavr Savr* tomatoes containing the selective marker were fed to rats. What is most conclusive of

all, is that NPT II gene has been infused into cancer patients as a part of **gene therapy**. What can be a better demonstration than this to show it has no effect on the human system?

Does NPT II hamper oral therapy using kanamycin and neomycin? The answer is a definite "no". After all NPT II is a protein which does not contain any unusual amino acids. Like any other protein in the daily diet it will be rapidly broken down in the acidic environment of the stomach and therefore become inactivated. Even if a small amount of NPT II escapes being broken down in the stomach it still would be ineffective in the gut, because NPT II requires adenosine triphosphate (ATP) as a source of energy for inactivating kanamycin and neomycin, and ATP is not available in the lumen of the gut and therefore the enzyme cannot function. Degradation of NPT II and its gene in the stomach and large intestines is also the reason why pathogenic bacteria which are largely located in the gut, cannot acquire antibiotic resistance by the transfer of the genes from the ingested transgenic plants.

There is fear that antibiotic resistance gene will be passed on to the microorganisms in the environment. Transmission in bacteria is mainly restricted to exchange of plasmids. While it is true that plasmids are used as vectors for introduction of the transforming gene and the marker, but during transformation they are integrated into the plant chromosome structure, before they express themselves. Since NPT II gene gets integrated into the chromosome it is highly unlikely that such a gene would get transferred to bacteria.

While many argue about the safety of antibiotic resistance as selective marker, two USDA scientists David Ow and Emily Dale have tackled the problem of NPT II in a more practical way. They have devised a protocol by which they snip out the NPT II gene before regenerating the plants from transformed cells.

It is very well that means have been devised to snip out marker genes, but what about genes that are actually re-



quired and yet produce a substance which is harmful? For example, the B t toxin gene of *Bacillus thuringiensis* which is incorporated into plants to confer insect resistance? B t endotoxins are not harmful to mammals. They are produced as insoluble crystals in the plant cells. On being ingested by insects they are broken down in their alkaline guts to the effective toxin. However, human beings and other mammals produce acid in their stomach, so the insoluble crystals are not converted into the harmful endotoxin. So plants containing B t toxin are harmless to mammals. It may also be pointed out that 'B t crystalline spore' is an effective and widely used natural insecticide. It is routinely used on vegetable crops to prevent attacks by caterpillars. To reach this state of commercial production and widespread use the insecticide has naturally been strictly screened for any potential toxicity to human beings and other animals.



But the environmental impact of B t toxin is yet to be thoroughly assessed. Does it affect the food chain? After all the plant eating insects that can break down the crystals to the active form of the toxin are eaten by birds and other animals. What is the fate of the toxin in such cases? Does it build up like DDT in the food chain? When B t toxin producing plants die and decay in the soil, particularly an acid soil, how are fauna affected? Many questions about B t toxin transgenic plants are yet to be investigated.

Ecologists in general have a lot of reservations about release of transgenic plants. While they are aware of the necessity of recombinant DNA technology to boost food production for the fast- growing human population. They have three major concerns.



They point out that the recombinant genes may escape via pollen and enter the DNA of weeds through hybridization and thus create new weeds or amplify the weed problem. Then again, the transgenic plants may escape cultivation and become weeds. Also, the use of certain transgenic crops may affect the soil and microorganisms and/or lead to agronomic practices generally harmful to the environment. The pros and cons of the above concerns are hotly debated among scientists. In the case of genes such as herbicide resistance, insect resistance and stress tolerance, the fear is of the traits being passed on to the weeds, making them harder to control.

Certain experiments carried out to assess the environmental safety of such plants suggest that transgenic plants should only be grown if there is spatial isolation from weeds with which they can crossbreed. Thus it would appear that each transgenic crop will have to be evaluated in each of the areas where it is normally cultivated before it is introduced. There is fear about the transgenic plants escaping and becoming a weed. Pessimists are quick to point out the inherent danger of introducing organisms into a new environment, citing the cases of European rabbits in Australia, muskrat in Europe. Africanized bees in South America and water hyacinth in the Indian subcontinent. Introduced with a good intention they have run wild and created havoc. The relevance of these examples are being challenged on the ground that in the new locales the organism lacked its biological controls which had coevolved with it in its natural habitat, and kept it from running wild. The 'invasiveness' of transgenic oil seed rape has been extensively studied in U.K. by M.J. Crawley and his colleagues. Invasiveness was defined by them as the rate of population increase of oil seed rape from one year to the next. The three year study clearly established that under none of the wide range of experimental conditions did the transgenic plants show different rate of population growth to those of their unmodified counterparts. However, critics have pointed out certain flaws and Crawley and his colleagues are also cautious in their appraisal of the results. They say, "It would be prudent' to reserve judgement on the risks that might be posed to other crop species or different transgenic constructs (such as transformations like drought tolerance or pest resistance, which might be expected to enhance plant performance in natural habitats)".

Some scientists feel that even herbicide resistant 'escapes' (crop plants which have turned wild) can be a danger in the immediate vicinity of the field. However, many others feel that this may not be the case as centuries of growing as



Is this to remain our only option?

cultivars leave these crops with little vigour to compete in the wild.

The third question being raised by ecologists is how far will these transgenic crops bring changes in agricultural practices. Herbicide resistance in crops will allow farmers to utilize larger quantities of herbicides which are eco-friendly. Transgenic plants incorporating disease and pest resistance will allow the farmer to cut down on pesticide use. Thus from this point of view transgenic plants should not only be a boon to

farmers but eco-friendly as well. What emerges from the debate is that assessed carefully for the various risks, transgenic plants have a bright future. While toxicity to human beings and animals can be routinely tested according to stringent guidelines; ecological safety is quite another matter. No guidelines are yet available to assess eco-friendliness of genetically engineered crops. The norms and guidelines however has to be stringently worked out if transgenic crops have to go beyond the labs and into the field so as to benefit the common man.



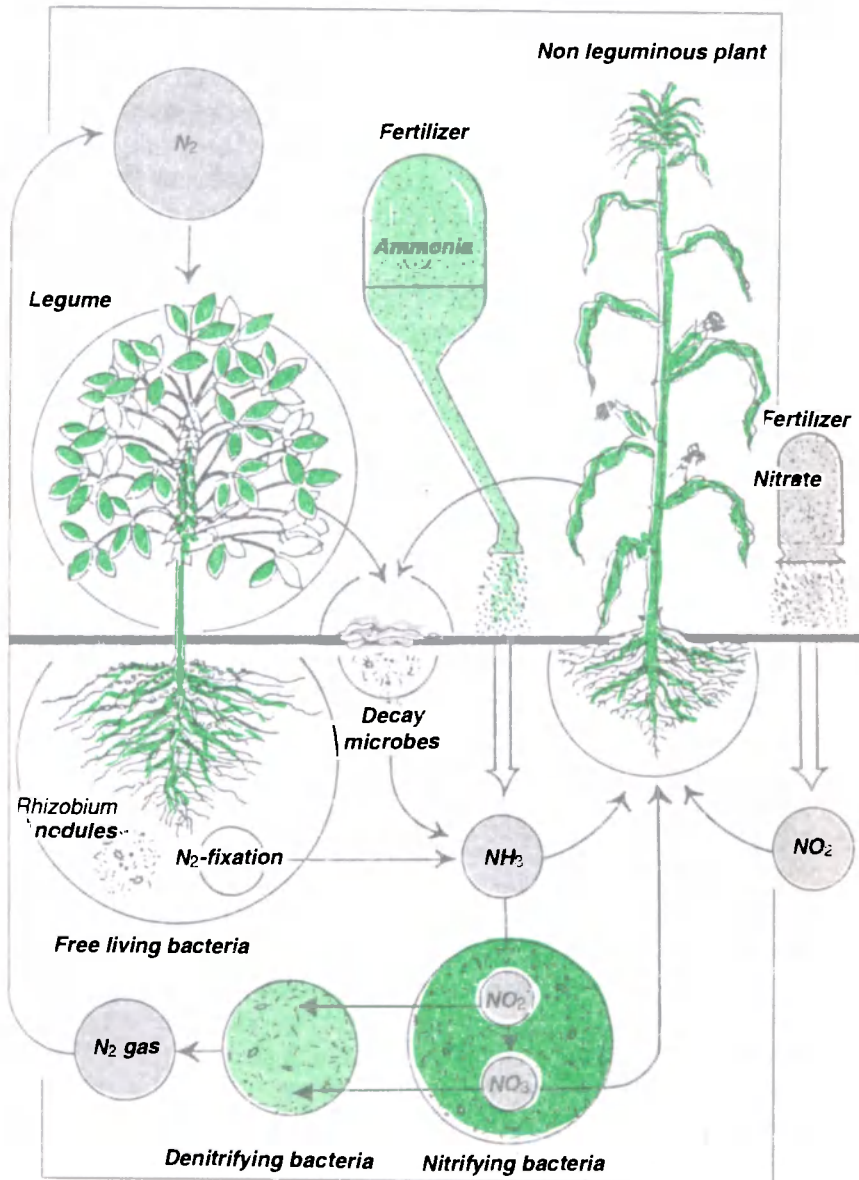
Even though agriculture started almost ten thousand years ago, the importance of mineral nutrition for plants came to be recognized only in the nineteenth century. By the middle of the century it had been established that certain elements including nitrogen, phosphorus and potassium were necessary for the healthy growth of plants. In 1840, a French scientist Jean-Baptiste Boussingault showed that plants obtain nitrogen from nitrates in the soil. About the same time, Samuel Dana described how phosphates present in the manure act as fertilizer, and John Bennet Lawes and J.H. Gilbert at the Rothamstead Experimental Station in England,



The natural way

discovered the fertilizer, superphosphate. In 1852, the first inorganic fertilizer, potassium sulphate, was produced in the Strassfurt mines, in Germany. During World War I, a Polish scientist, Edward Haber, working in Germany, discovered a chemical process by which atmospheric nitrogen was converted into ammonia by reaction with hydrogen at high temperature and pressure in the presence of a catalyst. Till today, the Haber-Bosch process is the sole one for the manufacture of nitrogenous fertilizers.

The advent of fertilizers was a boon to the farmers, who had solely depended on farm yard manure and rotation of crops to restore the fertility of the soil. The necessity to leave land fallow for a season to regenerate its fertility vanished. The importance of fertilizers increased still further with the advent of the high yielding varieties of crops. These crops demand high levels of nutrition failing which their yield is less than the traditional varieties. In India, the Green Revolution



Nitrogen cycle








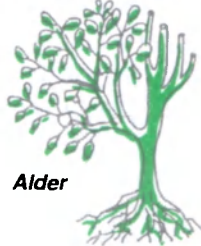
brought in its wake, massive use of chemical fertilizers leading to contamination of water by nitrates and increased salinity in the soil. Chemical fertilizers are very expensive from the energy point of view, leading to high cost and dependence on non-renewable energy sources. To this day, the Indian government has to subsidise the cost of fertilizers so that it is affordable for the farmer and does not increase the price of the food grain.

The focus has therefore shifted to biofertilizers. Simple biotechnologies facilitating plant nutrition include biological nitrogen fixation, use of phosphobacterium and mycorrhizae which are fungi that live in and on the roots of plants, earthworm rearing and biogas technology which produces, in addition to fuel gas, an organic residue extremely rich in minerals.

Nitrogen is a major component of proteins, nucleic acids and other important biological molecules. Even though air contains 80 per cent nitrogen, plants and animals cannot utilize it. Plants absorb nitrogen mainly as nitrates while animals require it in an organic form. The only organisms that can utilize atmospheric nitrogen are certain bacteria and cyanobacteria commonly known as blue-green algae.

The micro-organisms 'fix' nitrogen with the aid of an enzyme complex called nitrogenase. The nitrogenase enzymes reduce molecular nitrogen obtained from the atmosphere to produce ammonia. The energy is also supplied by the cell in the form of ATP. Thus, the biological process strongly resembles the Haber-Bosch process with nitrogenase acting as the catalyst, and fuel being replaced by ATP. Nitrogen-fixing micro-organisms are widely distributed in nature and fall into two classes — the free-living nitrogen fixers and the symbiotic nitrogen fixers which fix nitrogen only in association with plants.

Some of the free living nitrogen fixing bacteria such as *Azotobacter* require oxygen to flourish and others grow only

<i>Nitrogen-fixing organism</i>	<i>Associated organism</i>
 <i>Aabaena azollae</i>	 <i>Waterfern</i>
 <i>Nostoc muscorum</i>	 <i>Tropical herb</i>
 <i>Azospirillum lipoferum</i>	 <i>Tropical grass</i> <i>Corn</i>
 <i>Frankia alni</i>	 <i>Alder</i>

Members of the nitrogen-fixing club

in the absence of oxygen, for example, *Klebsiella*. *Rhodospirillum* can survive both with or without oxygen.

Among the large number of nitrogen-fixing, free-living bacteria, only two have attracted the attention of scientists in as far as nitrogen-fixation is concerned. They are *Azotobacter* and *Klebsiella*.

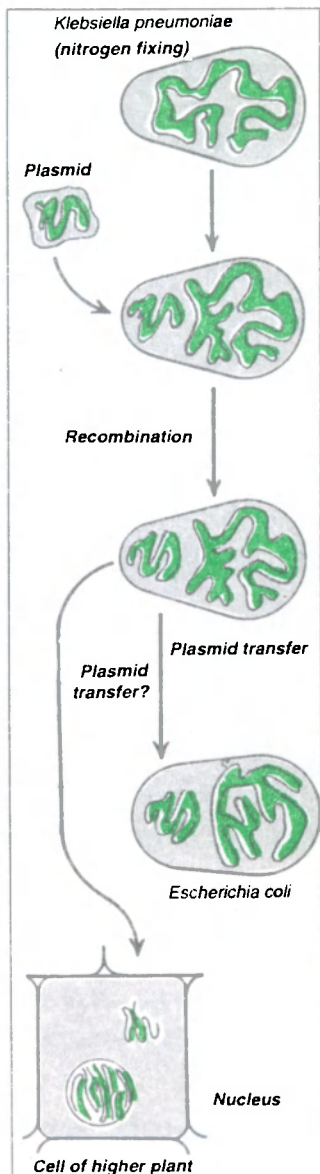
The factors which determine the growth of *Azotobacter* are, the amount of organic matter in the soil and the presence or absence of certain other micro-organisms. Besides fixing nitrogen *A. chroococcum* has the ability to synthesize and secrete into its environment, B vitamins, plant growth regulators and antifungal antibiotics. This may explain the beneficial effect of this organism on seed germination. *Azotobacter* cultures have been used to inoculate seeds and seedlings to increase growth. *Azotobacter*, however, has one great drawback. Its nitrogen-fixing ability is regulated by the presence of nitrogenous compounds in its environment. Thus, it cannot be used along with a chemical fertilizer. Attempts are being made to isolate mutants of *Azotobacter* which are not sensitive to external nitrogen and which will even excrete ammonia into its surroundings. Such a mutant can have a widespread use as a biofertilizer. One may even dream of a day when such mutants of *Azotobacter* grown in a bioreactor under controlled environmental conditions will supplant the high energy requiring Haber-Bosch process for the manufacture of nitrogenous fertilizers. Another bacterium which has drawn the attention of researchers is *Klebsiella*. It has been possible to isolate its nitrogen-fixing genes (*nif* genes) and to introduce them into *E. coli*. All of the 17 *nif* genes have been cloned and their products identified. It is hoped that one day it will be possible to introduce these genes into non-nitrogen fixing crop plants to make them self-sufficient for nitrogen.

The second group of free-living nitrogen fixers are the cyanobacteria, commonly called, blue-green algae. More than a hundred species of blue-green algae are nitrogen-fixing. Nitrogen fixation takes place in special cells called the

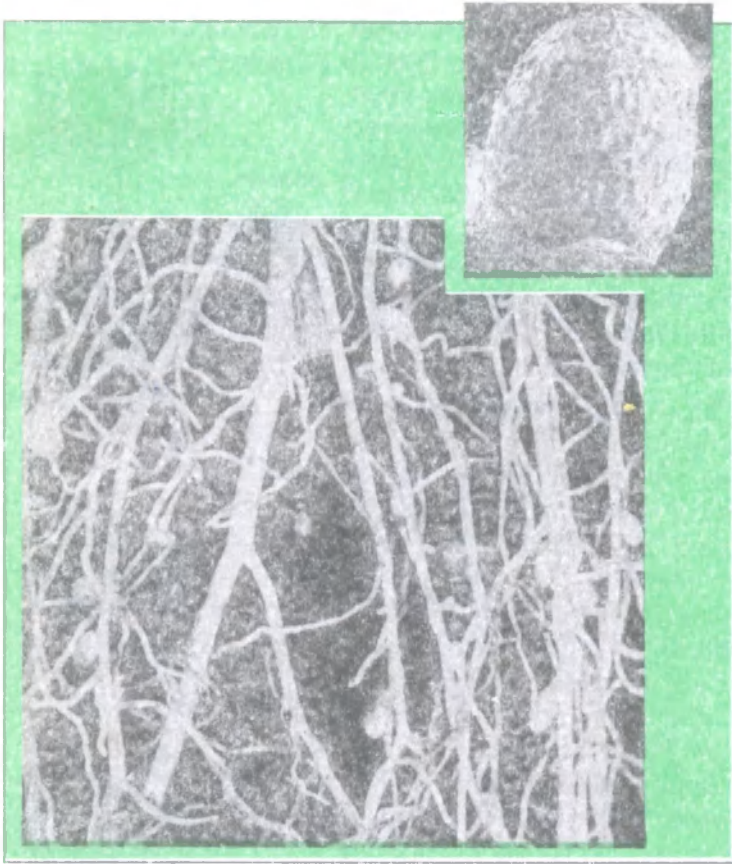
heterocysts which are large, thick-walled and apparently empty cells. The coloured vegetative cells and the heterocysts depend on each other for active nitrogen fixation, as the former supplies the energy for nitrogen fixation by the latter, while the fixed nitrogen is utilized by the photosynthetic cells for growth and development.

Blue-green algae are very common in the rice fields where they add to the nitrogen economy. If no chemical fertilizers are added, inoculation of the algae can result in 10-14 per cent increase in crop yields. Unlike *Azotobacter*, the blue-green algae are not inhibited by the presence of chemical fertilizers. On the other hand, presence of superphosphates accelerates their growth. The algal biofertilizer is particularly suitable for rice, as the crop is grown under flooded conditions.

Not only are algal fertilizers easy to produce, they are cheap. The cost of 10 kg may be only about Rs 30-40. Normally, continuous application for three to four consecutive seasons results in the build-up of an appreciable blue-green algae population in the field. The algal effect can be seen in the subsequent years



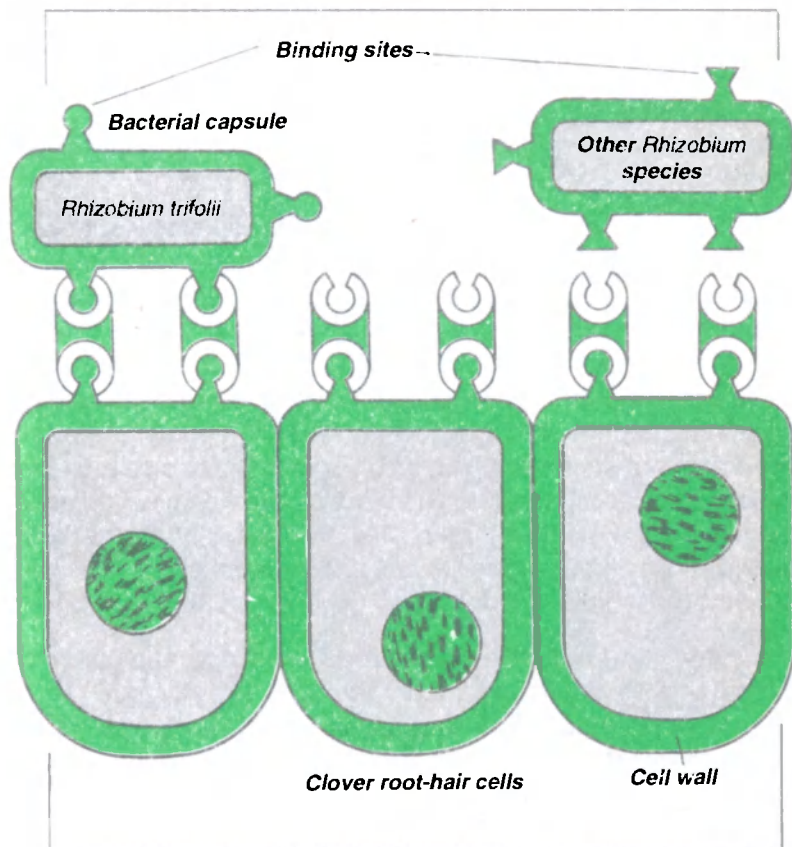
Nifty tailoring



Root nodules. Micrograph of root
nodule of clover (inset)

without the use of fresh inoculants. These algae have proved beneficial in the case of certain crops such as vegetables, cotton and sugarcane, when added along with chemical fertilizers.

Centuries before the understanding of plant nutrition, the ability of legumes to improve soil fertility was known. In the latter half of the nineteenth century, it was proved that nodules on the roots of legumes such as peas and beans are respon-



Joining hands

sible for the fixation of atmospheric nitrogen. In 1888, the Dutch scientist Beijerinck conclusively proved that, it was bacteria present in these nodules which are responsible for nitrogen fixation. These bacteria were called *Rhizobium*.

Rhizobia usually inhabit the soil. When legume seeds germinate, the rhizobia are attracted towards the roots. The next step is in response to something exuded by the bacteria. The root hairs located near the growing tip of the root, curl

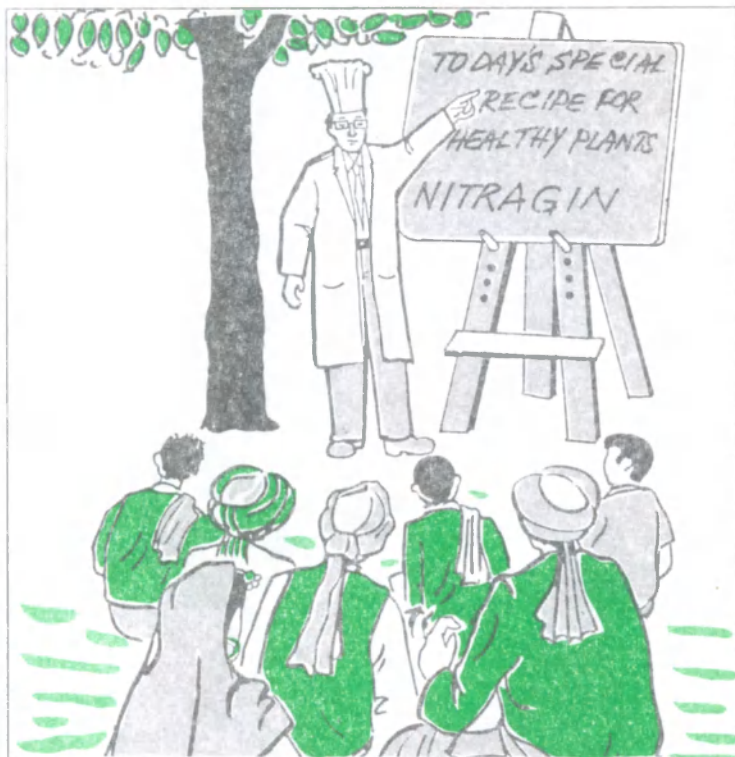
round the bacteria. The cell wall of the root hair grows inwards forming an 'infection thread' through which the bacteria reach the cells of the root. Here, the bacteria are released. The root cells become enlarged and differentiate into nodules. The bacteria inhabiting the cells transform into nitrogen-fixing bacteria.

Different species of *Rhizobium* infect different species legumes. There are seven groups of legumes — pea group, bean group, clover group, alfalfa group, lupin group, soybean group and the cowpea group — each infected by a different species. *R. phaseoli* which infects beans, does not infect peas or lentil, while *R. leguminosarum* which infects lentils, does not infect beans. If the soil in which a legume is grown does not contain the proper type of *Rhizobium* or else too few cells of the correct type, then nodule formation is inhibited and the plant does not fix nitrogen. The problem could be solved if *Rhizobium* of the proper type could be introduced into the soil when seeds are germinating.

As early as 1895, scientists introduced a laboratory grown culture of *Rhizobium* under the trade name of "Nitragin". The culture contained nodule extracts, gelatine, sugar and asparagine in a solid medium. Seventeen different types were marketed for different crops.

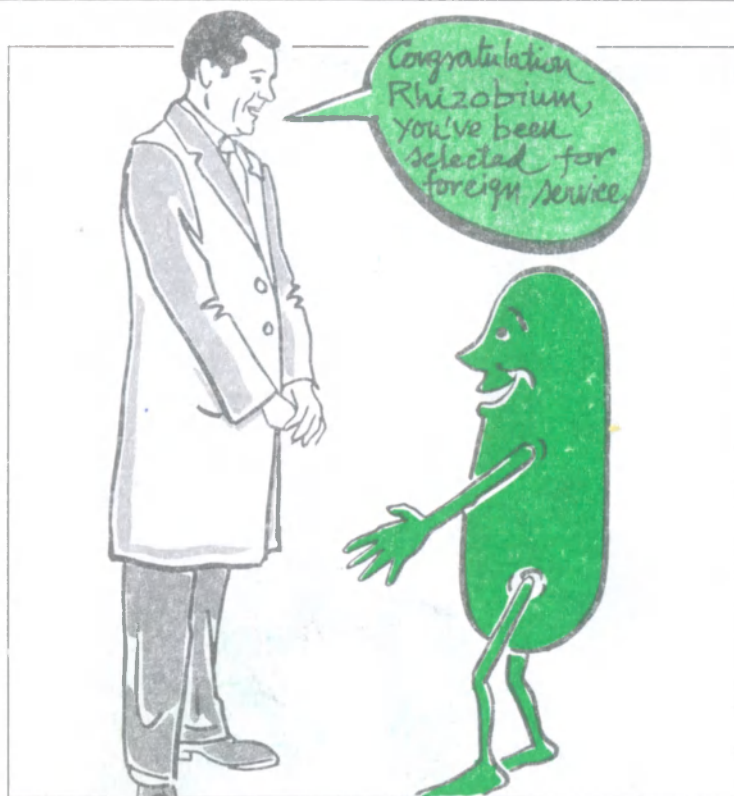
Rhizobial inoculations are used to this day, though the method of production is different. Pure cultures have replaced the nodule extract. Stock cultures of the seven different species are maintained. From this large broth, cultures are made, as required. *Rhizobium* is sold in two different forms — one as an inoculant to be used during seed sowing and second as pelleted seed.

The first attempts at genetic engineering of *Rhizobium* was to improve its performance. *R. meliloti* forms root nodules on alfalfa. Eighty per cent of the alfalfa seeds sold in the U.S.A. are pelleted with *Rhizobium*. Scientists at Biotechnica International at Cambridge, (U.S.A.) have engineered *R. meliloti*

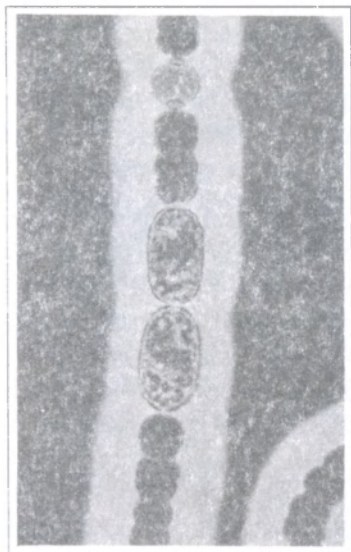


to produce more nitrogen. They have cloned the *nif* gene from the *Rhizobium* and reinserted a number of copies of this gene into the bacterium. The engineered strain increased the alfalfa production, by about 17 per cent.

There have been attempts, albeit not too effective, to introduce the *nif* genes into crops that normally do not fix nitrogen. Having failed in the direct transformation of cereal crops, scientists thought of inducing nodules in non-leguminous plants. It was not very difficult to induce *Rhizobia* to form nodules in important crops such as wheat, rice and oil seed rape. Scientist Edward Cocking believes that nodule formation and concurrent nitrogen fixation would be possible, if a way of introducing the *Rhizobium* into the roots could be



found. The cereal roots not having root hairs, offer a barrier to the entry of the bacterium. In order to facilitate the entry of the bacteria, the root tips were first treated with cellulose to dissolve the cell wall. Next, the treated roots were exposed to rhizobia in presence of polyethylene glycol. This detergent-like chemical dissolves the cell membrane, allowing the bacteria to enter. The scientists were happy to discover that nodules would form in cereals – but alas, the nodules did not fix nitrogen! However, the experiment has opened up hope that, some day it might be possible to have cereals with nitrogen-fixing nodules. Even though the major focus of research has been on *Rhizobium*-legume symbiosis, there is enough data to show that *Azospirillum* which inhabits both



Anabaena showing heterocysts

root cells as well as the surroundings of the root, increases the nitrogen-fixing potential of tropical forage grasses such as *Digitaria*, *Panicum*, *Brachiaria*, *Cyanodon* and cereals maize, sorghum, rice, wheat and rye.

Since nitrogen economy is positively affected by the presence of *Azospirillum*, methods have been developed to use it as a biofertilizer. When pelleted, the bacteria remain viable up to 31 weeks. In transplanted rice, seedlings dipped in a slurry of the inoculum, showed substantial fixation of nitrogen.

A major drawback to the regular use of *Azospirillum* for nitrogen fixation is the fact that, bacteria break down nitrates into nitrites and also that they break down nitrites into molecular nitrogen. Since plants absorb nitrogen mainly as nitrates, to make *Azospirillum*, a viable fertilizer, mutants have to be obtained which do not have the two deleterious effects.

Another symbiotic relationship which has been exploited as cheap biofertilizer is the *Azolla-Anabaena* complex. *Azolla* is a tiny water fern, common in ponds, ditches and rice fields. *Anabaena azollae* inhabits some of the cells on the underside of the fronds. It can fix nitrogen but is, however, dependent on the fern *Azolla* to provide it with photosynthates which supply the energy for nitrogen fixation.

Azolla has been used as a biofertilizer for rice in all major rice-growing countries of the world including India, Thailand, Korea, Philippines, Brazil and West Africa. The nitrogen

accumulated in the *Azolla* is made available to the rice crop when the fern decomposes. In addition to nitrogen, the decomposed *Azolla* also provides potassium, phosphorus, zinc and iron to the crop. Use of *Azolla* as biofertilizer has another beneficial effect. It successfully controls aquatic weeds which would otherwise compete with the crop for nutrients.

Rhizobia and cyanobacteria are not the only symbionts that aid in nitrogen fixation. *Frankia* form nitrogen-fixing nodules in eight groups of woody shrubs and trees. Cells of the root secrete substances that provide food for the bacteria. The latter, then invades the cells of a developing lateral root and causes it to turn into a nodule. Entry into the host plant changes the structure of the microorganism, as well as its metabolism. After entering the cell, the filaments branch repeatedly forming a dense cluster. The tips of the hyphae swell into large pear-shaped or round structures or compartments with thick walls. The thickening of the walls of the vesicles prevents oxygen from diffusing in and it is here that nitrogen fixation takes place. Scientists are hopeful that, some day they may be able to make fruit trees, such as apple, pear, plum, cherry, raspberry, apricot, and peaches, fix nitrogen through the involvement of *Frankia*.

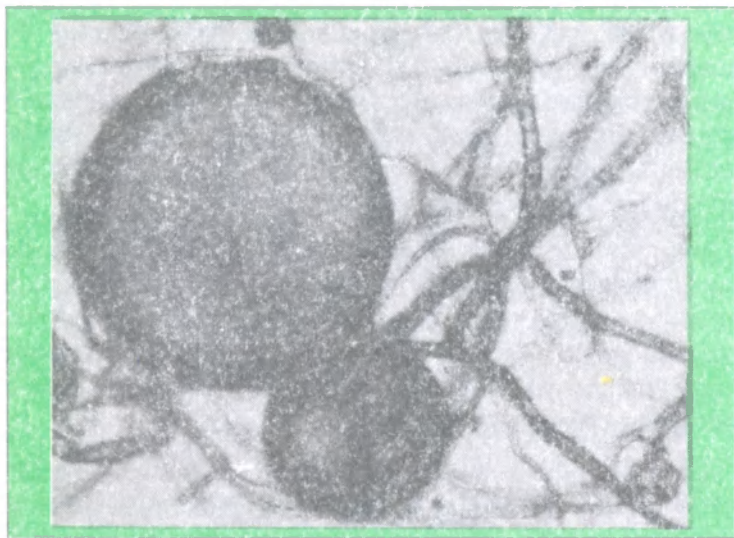
The soil is inhabited by a number of bacteria which if properly exploited can enhance its fertility. Amongst these are, a group of bacteria termed as phosphobacterium which have the capacity of releasing bound phosphate from the soil and thus making it available for the plants. They act in different ways but all ultimately increase phosphate availability in the soil. Some bacteria secrete organic acids such as lactic acid, acetic acid, citric acid, which solubilise the bound phosphates into forms available to plants. Others produce sulphuric acid by oxidation of sulphur, and this acid then acts like the organic acids do in making phosphates available to plants. The carbonic acid formed by the action of carbon dioxide released by the bacteria during respiration and water, acts in a similar way. Some micro-organisms give off hydro-

gen sulphide which reacts with the iron salt — ferrous phosphate — to form ferrous sulphide and thus releases the phosphate. Microbes are also responsible for the decay of dead animal and plant matter in the soil. During the decomposition, humic and fulvic acids are formed which bind the metal ions such as iron, aluminium, manganese and calcium and release the phosphate ions for the plants.

The most common varieties of phosphobacterium are *Pseudomonas* and *Bacillus megaterium*. The phosphobacterium has been marketed as “phosphobactrin”. Cultures similar to that of *Rhizobium* are available for use with seeds. In conjunction with *Rhizobium*, it gives a large increase in yield of legumes and other crops.

In the soil, the plant roots are not in a sterile environment. They are surrounded by organisms, some of which are ene-





The friendly underground fungal network

mies, others friends. Among the helpful organisms are, certain non-pathogenic fungi called mycorrhizae. One group of mycorrhizae forms a sheath round the fine roots. These help the plant by helping in solubilising nutrients near the plant roots and making it easy for the plants, to feed. Secondly, they prevent the roots from being attacked by nematodes, by entangling them.

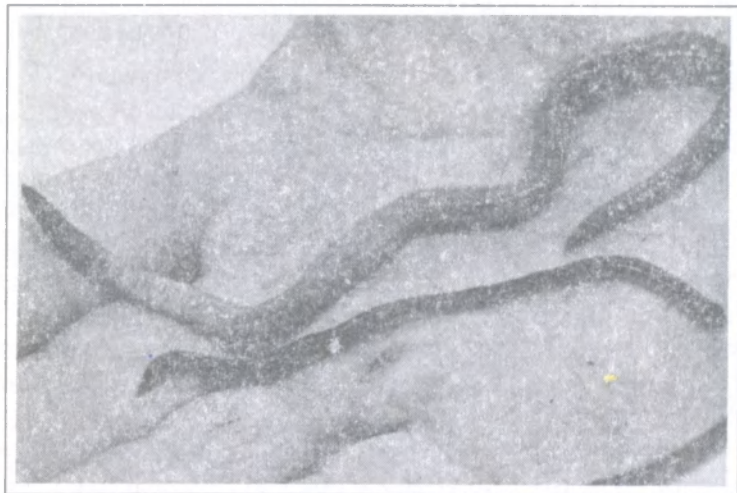
The second group penetrate the roots and establishes symbiotic relations with the plants. In this symbiosis, the fungi help the roots in obtaining inorganic nutrients while obtaining essential organic nutrients from them. How close and necessary this relationship is, can be seen from the example of the citrus. Soil in which citrus seedlings were growing, was fumigated with methyl bromide to kill pathogens. The seedlings, however, did not benefit from this treatment. Instead, they were stunted and many died. The cause was traced to

mycorrhizae which were also killed along with the pathogens. The plants suffered from the lack of symbiosis and died.

These mycorrhizae have existed in harmony with plants for millions of years as is apparent from the fossil records. Their discovery, however, is quite recent as they do not cause morphological changes in the roots. Thus, in earlier times, roots harbouring endomycorrhizae were thought to be uninfected. They are of particular significance in agriculture as they assist plants to absorb phosphates from the soil. Phosphate ions are not very mobile in the soil, thus when a plant uses up all the available phosphates near its roots, it starves for want of phosphorus. The mycorrhizal mycelia spread far and wide, away from the depleted zone and translocates the phosphate directly to the host. Plants with restricted root systems, having short stubby roots and few root hairs are particularly benefited by association with mycorrhiza. Absorption of elements which are required in minute quantities by plants are also facilitated by these mycorrhiza.

These mycorrhiza have another beneficial character. They are particularly valuable in times of drought. They enable plants to survive and grow in the driest and poorest of soils, even in leached soils that have been ruined by deforestation and overcropping. With such a high range of hosts and benefits, scientists have been trying to develop an inoculum which will have a fairly long shelf-life so that, like *Rhizobium*, it can be inoculated along with the seed.

Earthworms are known to be farmer's friends. They dig up and mix the soil for him, eat up decayed plants and convert them to fertilizer thus, enriching the soil. However, the continuous use of agricultural chemicals have in most cases depleted the soil of its natural rejuvenators, the earthworms. In recent years, a low-tech biotechnology has emerged to restore earthworms to their natural place in the environment. Vermiculture requires minimum inputs in terms of ingredients such as leaf litter, household and agricultural wastes along with of course, a starting population of earthworms.



Farmer's friends

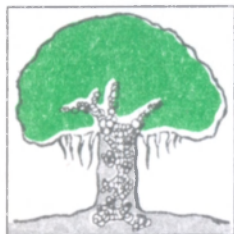
Energetic entrepreneurs are producing vermi-compost, by allowing earthworms instead of natural micro-organisms in the soil to convert the waste plant matter into compost. The major drawback for the purchaser is the fact that there is no way of standardizing the vermi-compost and unscrupulous producers may mix it in soil to dilute the content.

In our country, cow dung and agricultural wastes have been a source of either fuel or organic fertilizer. In both cases, the material is not utilized to its full potential. With the stress on renewable energy sources to supplant fossil fuel, the development of bio-gas plants have received a boost. In bio-gas plants, dung and agricultural wastes are fermented with the help of methane-producing bacteria. The gas evolved can be used for cooking and lighting. The residue which is semi-solid, is a rich source of minerals particularly, nitrogen. It can be dried and used as a fertilizer. This biofertilizer is on the market already, but not in large enough quantities, as the bio-gas project in our country has not done too well, mainly because of social constraints.

Though, in the near future, we may not be able to do away completely with chemical fertilizers which are expensive and eco-unfriendly, but we may be able to substitute them to a great extent with the relatively cheap and definitely eco-friendly biofertilizers.



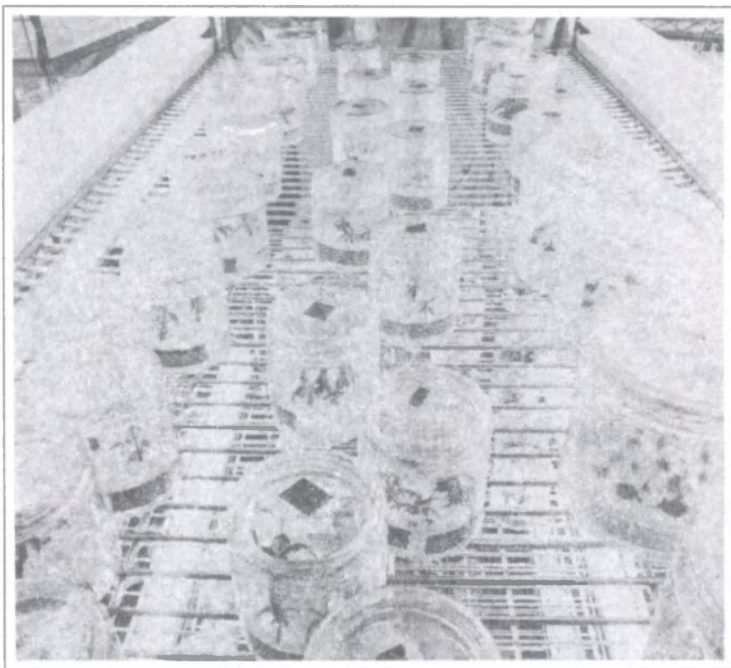
The twentieth century has seen a great change in agriculture. Starting with the period when farmers were the selectors of the varieties of crops to be cultivated, followed by the emergence of agriculture as a science and crop breeding in the hands of the scientists to the present when thanks to the rapid development of biotechnology it has become the domain of the molecular biologist. The century has also seen depletion of forests and arable lands due to population increase. What lies in the next century? A burgeoning population to be fed and clothed, a rapid depletion of non-renewable energy sources and fear of a highly polluted



Future perfect

earth. But human beings have so far managed to control nature to their own benefit and no doubt scientists will find a way out of the miserable scenario. So far as agriculture is concerned it would not be wrong to assume that genetic engineering will play a major part.

The molecular biologists of the present century have mainly been interested in transgenic plants which facilitate crop management such as insect and disease resistant varieties. Very few attempts have been made to improve crop quality. This is where the main stress will lie in the future. Some other advances would include changing the photosynthetic rate, changing the structure and quantity of the storage products such as balanced amino acids in the storage proteins, manipulation of nitrogen fixation and growth regulator mechanism, and environmental stress resistance. Some scientists are already trying to improve crop quality and to produce designer plants.



Futuristic orchards

Starches are an important component of our diet. It is also used in the manufacture of paper, board and textiles, besides acting as raw material for production of ethanol, fructose and gluconate. In most of these cases the starch obtained from the plant source has to be chemically modified before use. It would therefore be of great interest to produce them directly in the plants so as to obviate the need to chemically process starch. Most of the commercial starch is produced from maize, however, a substantial proportion of it is also produced from potato. Scientists are now trying to modify the quality and quantity of starch in potatoes. The first experiments of transgenic plants with increased starch production did not prove to be practical as the plant's metabolism could not cope with it. However, when the gene was so manipulated as to



only express in the tuber, the experiment yielded potatoes with high starch content. In countries where food is cooked in microwave ovens, high starch content makes for better quality of the product. The increased starch content also results in less oil being absorbed when potato chips are made, thus making them more suitable for consumption.

Introducing genes from wild plants that improve the quality of the product is one of the targets of the future. Two proteins monellin and thaumatin have been recently isolated from two African plants, serendipity and katemfe. These are many times sweeter than sugar. The gene for monellin has been isolated and introduced into lettuce and tomato. The protein accumulated in the leaf and fruit enhancing not only their



flavour but also their quality. It did not increase calories nor did it cause an imbalance in the amino acids.

During the early years of work on the *Agrobacterium tumefaciens* plasmid it was discovered that a part which gets incorporated in the plant tissue, the *ipt* gene, codes for cytokinin. Currently cytokinins are used by farmers for enhancing the branching of ornamental plants. Recently it has been shown that cytokinins applied to cereals promote grain setting. They also increase tolerance of plants to stresses. If the *ipt* gene could be introduced so as to express itself in the correct place at the correct time then one could probably substitute for external applications. But to do these types of gene-engineering, as well as that for photosynthesis and



nitrogen metabolism requires a thorough understanding of not only the pathways of metabolism but also how the change may affect the whole plant system.

Who has not delighted in myriad of gorgeously coloured flowers that bloom around us? Flower-breeders are always trying to develop exotic colours and forms. They dream of blue roses and black tulips. In the twenty first century their dreams may come true. In most flowers the colour is due to a small group of water soluble pigments — the anthocyanins. Anthocyanins are also present in many fruits and foliage. The spectrum is indeed awesome — ranging from pink to scarlet, magenta violet to delphinium and gentian blue. Three groups of this pigment can be identified, cyanidins which produce the pinks and reds, the delphinidins, the blue and pelargonidins the orange. There are also some white anthocyanins.

The biosynthesis of anthocyanin pigments has been worked out both genetically as well as biochemically in petunia a common garden plant. It has been possible to insert genes into petunia using *Agrobacterium tumefaciens*. Cyanidins and delphinidins are present in petunia, but the gene for orange pelargonidins are absent, that is it cannot convert the anthocyanin precursor into pelargonidins because it does not have the correct enzymes. Scientists then isolated a gene from maize which when inserted into petunia produced many pelargonidins resulting in a brick red coloration in petunia which was never known before. This pioneering experiment in manipulation of anthocyanin biosynthesis has opened up prospects for producing unusual flower colours.

In the last few years the potentiality of using transgenic plants for production of a wide range of health and industrial products has been recognised. Plants have been engineered to produce speciality chemicals and novel biopolymers. Scientists have been successful in producing human proteins in plants. Antibodies have been produced in tobacco, serum albumin in potato and tomato. Oil seed rape plants produce leu-enkephalin, a pain killer produced in the human brain. A California based biotech firm is planning to market a sun screen product which will contain the skin pigment melanin produced by transgenic tobacco plants.

There are many advantages in producing human and industrial proteins in plants. The first reason is the quantity that can be produced. While genetically engineered bacteria grown in bioreactors can produce grammes of a required product, transgenic plants should be able to produce kilograms per hectare. The cost of production is low, as no sophisticated fermenters or continuous energy supply is required. Instead with the correct transgenic plants and standard agricultural inputs such as water, fertiliser and sunlight the desired products may be harvested easily. The price of such products would be much lower and therefore affordable. Another advantage of plant-based systems is that many



Production plants



mammalian proteins undergo modifications such as addition to sugar residues. Proteins made by bacteria lack these sugars whereas plants can carry out the modifications much more naturally.

Serendipity has played a role in the discovery that plants could make animal proteins. In 1918, Gus de Zoeten and Thomas Hohn introduced the gene for human interferon (a protein with anti viral activity) into turnip plants, hoping to make them resistant to viral infections. The plants did not develop resistance, but to the surprise of the researchers these plants did produce large quantities of interferon, and what was more important the interferon was active when given to animals.

About the same time Andrew Hiatt and his co-workers at the Scripps Institute in California were trying to produce tobacco plants with resistance to Tobacco Mosaic virus (TMV). Their strategy was to make the plant produce an antibody against these pathogens much the same way as

humans fight germs. While Hiatt and his colleagues are yet to develop resistant plants they have succeeded in producing plants which synthesise mouse antibodies. These plant made antibodies or 'plantibodies' as the discoverers call them, behave just like mouse antibodies *in vitro*.

The commercial prospect of producing antibodies in plants is immense. The first monoclonal antibody (a single molecular antibody species that binds to a specific site on an antigen) was produced by Georges Kohler and Caesar Milstein at MRC Laboratories in Cambridge, (U.K.), in 1975 by fusing a cancer cell with a normal blood cell. This complicated and expensive procedure is still the only method of producing monoclonal antibodies, which are now widely used as therapeutic agents as well as a diagnostic tool. However, the drugs and therapeutics may require some time to come to the market, as they have to be tested and cleared by Food and Drug Administrations before they are allowed to be marketed.

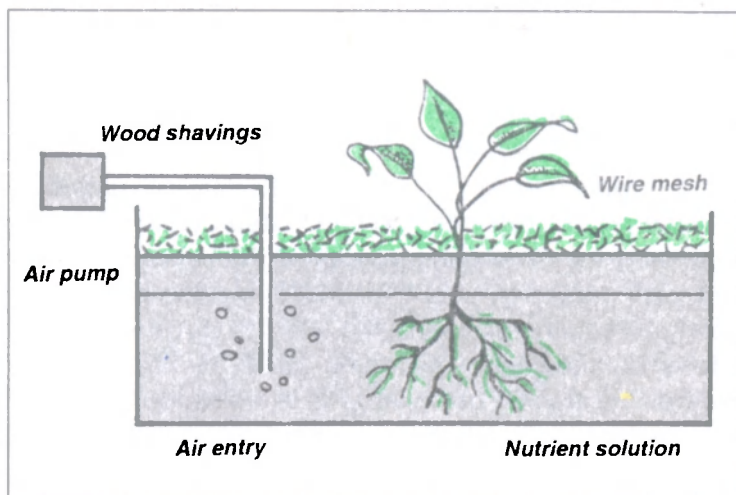
There is also wide scope for using transgenic plants for producing enzymes required for food processing and other industries, biopolymers and industrial oils. Lauric acid which is used in large quantities in detergents is currently obtained from palm and coconut which are hand harvested. It would be advantageous to produce it in field crops to facilitate harvesting. Calgene scientists in U.S.A. have been able to produce two different oils with unique composition which will be of value in food and detergent industries respectively. These have been expressed in transgenic rape. They are now attempting to engineer oilseed rape to produce jojoba oil, which is very valuable because it has a wide range of uses starting with cosmetics to transmission fluid and other lubricants.

We tend to think of plastics as synthetic substances, however, polyhydroxybutyrate (PHB) has been obtained from the bacterium *Alcaligenes eutropus*. The relevant genes from bacteria have introduced into *Arabidopsis* by scientists at

Michigan State University, U.S.A. Luckily the transgenic plants produced the PHB granules without killing the plant.

A large number of enzymes used in industry are manufactured in bioreactors using bacteria and algae. The highest demand is for starch degrading enzyme amylase which is used in the food industry. That transgenic tobacco plants can make the protein has been demonstrated by the Dutch firm Mogen and the American biotech firm Biosource Genetics. What was promising was that the transgenic plants grew vigorously, and the plant enzyme was just as effective as its bacterial counterpart in degrading starch. But the best surprise was the stability of the enzyme in the seed. This means that a natural long-lasting packaging was available — and experiments showed that milled seeds could be used as effectively as purified enzymes, thus leading to a substantial saving in labour as well as costs. This pioneering experiment opens up possibilities of producing other commercial enzymes at low cost in field crops and storing them as seeds.

The advent of the 21st century will not only see plants as factories but also factories that will produce plants. Green house or glass house technology is growing by leaps and bounds. Methods of cultivating plants in aqueous solutions is a very old one. There are mentions of the technique in *Rig Veda*. The Hanging Gardens of Babylon, one of the seven wonders of the ancient world was another example of the culture of plants under artificial conditions. Today commercial green houses use the nutrient flow technique (NFT) by which plants are grown with their roots in flowing nutrient solution, with or without solid support medium. NFT assures that the roots are well aerated for healthy growth. It also helps in minimising water usage as the same water is recycled, with the nutrients being monitored and replenished as and when necessary. This system along with controlled light and carbon dioxide concentration leads to yields far higher than that which can be obtained from field crops.



Growing plants in water

In many countries such as Israel, Holland and U.S.A. most of the tomatoes, lettuce and cucumber are grown in green houses and are thus available fresh the year round. Though the infrastructure requires large investments, returns are high. Green house yield of tomatoes are 450 tonnes per acre compared to 220 tonnes when grown in the field. In addition it is cheap labour wise and saves on water and nutrients.

With burgeoning population arable land will be at a premium. Many scientists predict that in the 21st century about a quarter of the world's food supply will be grown hydroponically. Some even go further and envisage a refrigerator size cabinet in the kitchen from which the house wife can get her daily requirement of fresh vegetables, and indeed some fruits, in not too distant a future.

Thus the ingenious human species in the 21st century may look forward to a time of gene revolution and green techniques as factories give way to plants and plants are grown in factories.



Glossary

Agar: A substance obtained from sea weeds. It forms a jelly and is an important constituent of tissue culture medium on which bacteria or plantlets are grown.

Antibiotics: Substances produced by a microorganism which inhibit the growth of other microorganisms.

Biological diversity: The great variety of life on earth which represents the great diversity of DNA, the molecule of life.

Callus: An unorganized proliferative mass of plant cells.

Clonal: Pertaining to cloning, a procedure for the asexual propagation of plants that are considered to be genetically uniform and originated from a single explant or an individual.

Gall: An unnatural growth on plants usually caused by young larvae of insects, but also caused by bacteria, fungi, molds and nematodes.

Gene therapy: The manipulation of DNA to provide effective control over certain genetic defects.

Genetic engineering: Technique for altering the genetic make-up of an organism to artificially endow it with desirable traits.

Hyphae: Fungal filaments.

Recombinant DNA: DNA which contains sequences from different sources, combined usually as a result of laboratory procedures *in vitro*.

Totipotency: The capacity (usually dormant) of a single cell to give rise to an entire organism.

SPECIAL efforts made by prehistoric people to domesticate and improve wild plants led to the formalization of agricultural science. Yesterday's spadework is reflected in today's lush green agrarian fields that feed the world. Biotechnology the tool to a better tomorrow, is at the forefront of attempts being made to create a greener world by conserving and harnessing biodiversity to give higher yields, increased pest resistance, better nutritional value and enhanced taste in cultivated crops.

This lucidly written and lavishly illustrated book provides fascinating glimpses into a future made hunger free by the yeoman service rendered by scientists manipulating the 'green gene'.

About the Author

Dr (Mrs) Shakuntala Bhattacharya (b.1937) did her D. Phil in Botany from Calcutta University. Her areas of specializations include Plant physiology and biochemistry and Radiation Biology. She has held various teaching and R&D assignments in India and abroad. She has always been interested in science education including curriculum development and teacher training.

Mrs Shakuntala Bhattacharya is widely travelled and has visited the USA, Australia, UK, Germany, France, Netherlands, former Yugoslavia, Malaysia and Philippines in connection with her research work.

She is the author of two textbooks in biology and twenty eight research papers and articles. **Green gene** is her first popular science book.

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